THE MALAYSIAN JOURNAL OF MedicalSciences

Volume 18, No. 4, 2011
ISSN 13-94-195X | e-ISSN 2180-4303

PENERBIT USM
Penerbit Universiti Sains Malaysia

Abstracts are indexed in:

refine your research SCOPUS™ ELSEVIER INDEX COPERNICUS INTERNATIONAL
EBSCO HOST Bioline International BRITISH LIBRARY

and 21 other international and Malaysian database

Editorial

Published by
PENERBIT UNIVERSITI SAINS MALAYSIA
Bangunan D34, Universiti Sains Malaysia
11800 USM, Pulau Pinang, Malaysia

Printed by
SINARAN BROS SDN BHD
389, Lebuh Chulia
10200, Pulau Pinang, Malaysia

List of Reviewers 2011

MJMS would like to acknowledge the people listed below for their contribution as reviewers in 2011. Their efforts tremendously help to improve the quality of submitted manuscripts.

No.	First Name	Last Name	Affiliation	Country
1	Nizar	Abd Jalil	Universiti Sains Malaysia	Malaysia
2	Nor Ba'yah	Abdul Kadir	Universiti Kebangsaan Malaysia	Malaysia
3	Faraizah	Abdul Karim	National Blood Centre	Malaysia
4	Ahmed Sahib	Abdulamir	Universiti Putra Malaysia	Malaysia
5	Mohamed Rusli	Abdullah	Universiti Sains Malaysia	Malaysia
6	Mohd Shafie	Abdullah	Universiti Sains Malaysia	Malaysia
7	Sarimah	Abdullah	Universiti Sains Malaysia	Malaysia
8	Liza Sharmini	Ahmad Tajuddin	Universiti Sains Malaysia	Malaysia
9	Abdulwahab A	Ajape	University of Ilorin Teaching Hospital	Nigeria
10	Sitara SR	Ajjampur	Christian Medical College	India
11	Salah	Al Awaidy	Ministry of Health	Oman
12	Adnan I	Al-Hindi	Islamic University of Gaza	Palestine
13	M Hesham	Al-Mekhlafi	University of Malaya	Malaysia
14	Redhwan Ahmed	Al-Naggar	Management and Science Universiti	Malaysia
15	Dimitrios	Andreadis	University of Thessaloniki	Greece
16	Pedro	Aqueveque	University of Concepcion	Chile
17	Maria Clara	Arbelaez	Muscat Eye Laser Center	Oman
18	Karthik	Arumugam	Advinus Therapeutics Private Limited	India
19	Mohamed Saufi	Awang	Universiti Sains Malaysia	Malaysia
20	Olutosin A	Awolude	University College Hospital, Ibadan	Nigeria
21	DO	Awonuga	Federal Medical Centre	Nigeria
22	Amanda	Azman	Center for Disease Control and Prevention	USA
23	Abdul Aziz	Baba	Universiti Sains Malaysia	Malaysia
24	Norsa'adah	Bachok	Universiti Sains Malaysia	Malaysia
25	Manjeshwar Shrinath	Baliga	Father Muller Medical College	India
26	Karim	Ben Ayed	University of Centre	Tunisia
27	Rosa Maria	Bermudez-Cruz	National Polytechnic Institute	Mexico
28	Anna	Berti	AO S Chiara	Italy
29	Komal	Bhambore	Nourish n Cherish	India
30	Biswa Mohan	Biswal	Universiti Sains Malaysia	Malaysia
31	Sabine	Bohnet-Joschko	Witten-Herdecke University	Germany
32	Azam	Bolhassani	Pasteur Institute of Iran	Iran
33	Marco	Bontempi	Rizzoli Orthopaedic Institute	Italy
34	Francesca	Borelli	University of Naples Federico II	Italy
35	Paolo	Boscolo-Rizzo	University of Padua	Italy
36	Kasonde	Bowa	University of Zambia	Zambia
37	Barbara	Caffery	Yorkville Eye Clinic	Canada
38	Ali	Celiksoz	Cumhuriyet University	Turkey
39	Ali Osman	Ceribasi	Fırat University	Turkey
40	Kausiki	Chakrabarti	National Institute of Immunology	India
41	Mushtaq	Chalkoo	Government Medical College, Srinagar	India
42	Sook Ching	Chan	Universiti Kuala Lumpur–Royal College of Medicine Perak	Malaysia

No.	First Name	Last Name	Affiliation	Country
43	Boon Hock	Chia	Tanglin Medical Center	Singapore
44	Marcia EL	Consolaro	State University of Maringa	Brazil
45	Nuno	Cortez-Dias	University Hospital Santa Maria	Portugal
46	Marco A	Cura	University of Texas Health Science Center	USA
47	Chuan Chao	Dai	Nanjing Normal University	China
48	Srijit	Das	Universiti Kebangsaan Malaysia	Malaysia
49	Heitor Franco, Jr	de Andrade	University of Sao Paulo	Brazil
50	Mary Anne Sampaio	de Melo	Federal University of Ceara	Spain
51	Jose Luis	Del Castillo Pardo de Vera	University Hospital La Paz	Spain
52	E Valdes	Dieguez	Hospital Universitario Central de Asturias	Spain
53	Prahlad	Duggal	Sri Guru Ram Das Institute of Medical Sciences & Research	India
54	Emmanuel C	Ejim	University of Nigeria Teaching Hospital	Nigeria
55	Nasser Attia	Elhawary	Umm Al-Qura University	Saudi Arabia
56	Mohamed Meselhy	Eltoukhy	Suez Canal University	Egypt
57	Zunaina	Embong	Universiti Sains Malaysia	Malaysia
58	Ding Zhi	Fang	Sichuan University	China
59	Giovanni	Fazio	University La Sapienza	Italy
60	Mark Simon	Ferguson	Barts and the London NHSTrust	UK
61	Imma	Ferrer	University of Colorado	USA
62	Valerie A	Ferro	University of Strathclyde	UK
63	Saini Jeffery	Freddy Abdulllah	University of Malaya	Malaysia
64	Hanne	Frederiksen	Copenhagen University Hospital	Denmark
65	Adediwura A	Fred-Jaiyesimi	Olabisi Onabanjo University	Nigeria
66	Rosa	Fregel	University of Las Palmas de Gran Canaria	Spain
67	Fioravante	Frischknecht	Kantonsspital St. Gallen	Switzerland
68	Endalamaw	Gadisa	Armauer Hansen Research Institute	Ethiopia
69	Chirag	Gandhi	University of Medicine and Dentistry of New Jersey	USA
70	Ramaswamy	Ganesh	Kanchi Kamakoti CHILDS Trust Hospital	India
71	Xu	Gao	General Hospital of Shenyang Military Command	China
72	Livio	Garattini	Centre of Health Economics	Italy
73	Michael A	Gbadegesin	University of Ibadan	Nigeria
74	Antonio	Giambona	Azienda Ospedaliera Vincenzo Cervello	Italy
75	Mahesh	Goenka	Apollo Gleneagles Hospital	India
76	Oscar D	Gomez Beltran	Reina Sofia Teaching Hospital	Spain
77	Jose Maria	Gonzalez Rave	University of Castilla La Mancha	Spain
78	Amit Kumar	Gorai	Birla Institute of Technology	India
79	Ankur	Goyal	All India Institute of Medical Sciences	India
80	Soraya M	Grossmann	Universidade Federal de Minas Gerais	Brazil
81	Carla Vecchione	Gurgel	University of Sao Paulo	Brazil
82	Ahmet	Guzel	Trakya University	Turkey
83	Habsah	Hasan	Universiti Sains Malaysia	Malaysia
84	Amiyakumar	Hati	Calcutta School of Tropical Medicine	India
85	Stephen P	Hawser	IHMA Europe Sàrl	Switzerland

No.	First Name	Last Name	Affiliation	Country
86	Dave	Heber	University of California, Los Angeles	USA
87	Nir	Hirshoren	Hebrew University School of Medicine–Hadassah Medical Center	Israel
88	Lin-Fen	Hsieh	Shin Kong Wu Ho-Su Memorial Hospital	China
89	Bing	Hu	Shanghai University of Traditional Chinese Medicine	China
90	Sun	Huh	Hallym University	Korea
91	Samsinah	Hussain	University of Malaya	Malaysia
92	Zabidi	Hussin	Universiti Sains Malaysia	Malaysia
93	Hayley	Hutchings	Swansea University	UK
94	Badrisyah	Idris	Universiti Sains Malaysia	Malaysia
95	Zamzuri	Idris	Universiti Sains Malaysia	Malaysia
96	Shatriah	Ismail	Universiti Sains Malaysia	Malaysia
97	Maria Immaculata	Iwo	Bandung Institute of Technology	Indonesia
98	Hasnan	Jaafar	Universiti Sains Malaysia	Malaysia
99	Mohammed	Jabr	Canadian Agency for Drugs and Technologies in Health	Canada
100	Ganesh C	Jagetia	Kasturba Medical College	India
101	Sachin	Jain	BR Nahata College of Pharmacy	India
102	Win Mar @ Salmah	Jalaluddin	Universiti Sains Malaysia	Malaysia
103	Sue	Jenkins	Curtin University	Australia
104	Viggo	Jonsson	Oslo University Hospital	Norway
105	Edmundas	Kadusevicius	Kaunas Medical University	Lithuania
106	Orawan	Kaewboonchoo	Mahidol University	Thailand
107	Surintorn	Kalampakorn	Mahidol University	Thailand
108	JM	Kamau	Botswana College of Agriculture	Botswana
109	George Em	Karniadakis	Brown University	USA
110	S Ananth	Karumanchi	Harvard Medical School	USA
111	Jurgen	Kasper	University of Hamburg	Denmark
112	Supaletchimi Gopal	Katherason	Universiti Sains Malaysia	Malaysia
113	Shellie L	Keast	University of Oklahoma	USA
114	Mohammad AH	Khan	Bangladesh Agricultural University	Bangladesh
115	Rahmatullah	Khan	Universiti Pendidikan Sultan Idris	Malaysia
116	Teik Beng	Khoo	Hospital Kuala Lumpur	Malaysia
117	Hashem Adnan	Kilani	Sultan Qaboos University	Oman
118	Daniel	Kimani	Centes for Disease Control and Prevention	Nairobi
119	Ismail	Kiran	Eskisehir Osmangazi University	Turkey
120	Beat	Knechtle	University of Zurich	Switzerland
121	Grace	Kollannoor-Samuel	University of Connecticut	USA
122	Haruki	Komatsu	Eastern Yokohama Hospital	Japan
123	Ta-Shen	Kuan	National Cheng-Kung University	Taiwan
124	Pim	Kuipers	Griffith University	Australia
125	Fenghua	Lan	Fujian General Hospital	China
126	Mostafa	Langarizadeh	Universiti Putra Malaysia	Malaysia
127	Lukas	Lee	National Health Research Institute	Taiwan
128	Chee Onn	Leong	International Medical University	Malaysia
129	Penny	Lewthwaite	University of Liverpool	UK

No.	First Name	Last Name	Affiliation	Country
130	Guoliang	Li	Vanderbilt University	USA
131	Choong-Chin	Liew	Harvard Medical School	USA
132	Yun-Wei	Lin	National Chiayi University	Taiwan
133	Hai-Shu	Lin	National University of Singapore	Singapore
134	Megan L	Lloyd	University of Western Australia	Australia
135	Andreas	Lopata	RMIT University	Australia
136	Andrew	Lozen	Froedtert Hospital	USA
137	Chao Bao	Luo	Taipei Veterans General Hospital	Taiwan
138	Manoharan	Madhavan	Saveetha Medical College	India
139	Nurahan	Maning	Hospital Raja Perempuan Zainab II	Malaysia
140	Niina	Matikainen	Helsinki University Central Hospital	Finland
141	Siyazi	Mda	University of Limpopo	South Africa
142	Naveen	Mehrotra	Robert Wood Johnson University Hospital	USA
143	Hui	Meng	Third Military Medical University	China
144	Dragan	Mirkov	University of Belgrade	Russia
145	Hasim	Mohamad	Hospital Raja Perempuan Zainab II	Malaysia
146	Mafauzy	Mohamed	Universiti Sains Malaysia	Malaysia
147	Mohamed Ashraf	Mohamed Daud	Universiti Sains Malaysia	Malaysia
148	Mohamed Izham	Mohamed Ibrahim	Qassim University	Saudi Arabia
149	Nor Azlin	Mohamed Ismail	Universiti Kebangsaan Malaysia Medical Centre	Malaysia
150	Khaled Mohamed	Mohamed Koriem	National Research Centre	Egypt
151	Abdul Aziz	Mohamed Yusoff	Universiti Sains Malaysia	Malaysia
152	Salmiah	Mohd Ali	Universiti Teknology Mara	Malaysia
153	Norazmi	Mohd Nor	Universiti Sains Malaysia	Malaysia
154	Shabnam	Montazeri	Family Research Institure	Canada
155	Phillip Edgar	Morgan	King's College Hospital	UK
156	Hussain Imam	Muhammad Ismail	Kuala Lumpur General Hospital	Malaysia
157	Kamarul Imran	Musa	Universiti Sains Malaysia	Malaysia
158	Norlaila	Mustafa	Universiti Kebangsaan Malaysia Medical Centre	Malaysia
159	Muzaimi	Mustapha	Universiti Sains Malaysia	Malaysia
160	Raju S	Muthu	Defence Institute of Physiology and Allied Sciences	India
161	John C	Muthusami	Christian Medical College	India
162	Sajan Jiv Singh	Nagpal	All India Institute of Medical Sciences	India
163	Lin	Naing	Universiti Brunei Darussalam	Brunei
164	Mukeshkumar	Nariya	Gujarat University	India
165	Nik Hisamuddin	Nik Ab Rahman	Universiti Sains Malaysia	Malaysia
166	Nik Hazlina	Nik Hussain	Universiti Sains Malaysia	Malaysia
167	Piyarat	Nimpitakpong	Naresuan University	Thailand
168	Rahmah	Noordin	Universiti Sains Malaysia	Malaysia
169	Rusli	Nordin	Monash University	Malaysia
170	D Olusoga	Ogbolu	Ladoke Akintola University of Technology	Nigeria
171	E Oluwabunmi	Olapade-Olaopa	University of Ibadan	Nigeria
172	Rokiah	Omar	Universiti Kebangsaan Malaysia Medical Centre	Malaysia
173	Valentine U	Omoja	University of Nigeria	Nigeria
174	Baafuor	Opoku	Kwame Nkrumah University of Science & Technology	Ghana

No.	First Name	Last Name	Affiliation	Country
175	Best	Ordinioha	University of Port Harcourt Teaching Hospital	Nigeria
176	Margaret	O'Rourke	University College Cork	Ireland
177	Nor Hayati	Othman	Universiti Sains Malaysia	Malaysia
178	Daniel U	Owu	University of West Indies	Jamaica
179	Karel	Pacak	National Institute of Child Health and Human Development	USA
180	John J	Palmieri	Massachusetts General Hospital	USA
181	Sunanda	Panda	Devi Ahilya University	India
182	Philip N	Patsalos	University College London	UK
183	Raj Kumar	Paul	Rajiv Gandhi Technological University	India
184	Wilfred	Peh	Alexandra Hospital	Singapore
185	Claudina	Perez-Novo	Ghent University	Germany
186	Aree	Pethleart	Thammasat University	Thailand
187	James Ian	Phillips	University of the Witwatersrand	South Africa
188	Sakorn	Pornprasert	Chiang-Mai University	Thailand
189	Saxby	Pridmore	University of Tasmania	Australia
190	Firdausi	Qadri	International Centre for Diarrhoeal Disease Research	Bangladesh
191	Betzabet	Quintanilla-Vega	National Polytechnic Institute	Mexico
192	Fatemeh Nabavizadeh	Rafsanjani	Tehran University of Medical Sciences and Health Services	Iran
193	Md Mizanur	Rahman	Universiti Malaysia Sarawak	Malaysia
194	Rajagopalan	Raman	University of Malaya	Malaysia
195	Giuseppe	Rizzo	University of Rome Tor Vergata	Italy
196	Benjamin Alexander	Rogers	University of Queensland	Australia
197	Mark	Rosenfield	State University of New York	USA
198	Antonio	Rubio del Campo	Universidad de Castilla-La Mancha	Spain
199	Primuharsa Putra	Sabir Husin Athar	KPJ Seremban Specialist Hospital	Malaysia
200	Nutjaree	Saejiw	Walailak University	Thailand
201	Sushma	Sagar	All India Institute of Medical Sciences	India
202	Siti Norlina	Said	Hospital Sultan Ismail	Malaysia
203	Khalid H	Sait	King Abdulaziz University	Saudi Arabia
204	Carolyn M	Salafia	St Luke's Roosevelt Hospital	USA
205	Ratana	Sapbamrer	Naresuan University Phayao	Thailand
206	Luis	Sarabia	University of Chile	Chile
207	Vijaya	Sarathi	Seth GS Medical College	India
208	Sreenivasan	Sasidharan	Universiti Sains Malaysia	Malaysia
209	Kittisak	Sawanyawisuth	Khon Kaen University	Thailand
210	Gary L	Schwartz	Mayo Clinic	USA
211	Rodney J	Scott	University of Newcastle	Australia
212	Shamala Devi	Sekaran	University of Malaya	Malaysia
213	Viplove	Senadhi	Sinai Hospital	USA
214	Cem	Sensogut	Dumlupinar University	Turkey
215	Ekrem	Senturk	Adnan Menderes University	Turkey
216	Qaisar A	Shah	University of Minnesota	USA
217	Mostafa Abbas	Shalaby	Cairo University	Egypt
218	Shirish S	Sheth	Breach Candy Hospital	India

No.	First Name	Last Name	Affiliation	Country
219	Rashidah	Shuib	Universiti Sains Malaysia	Malaysia
220	Ebby George	Simon	Christian Medical College	India
221	Rikki	Singal	Kundan Lal Hospital	India
222	Onkar	Singh	Bhopal Memorial Hospital and Research Centre	India
223	Rita S	Sitorus	University of Indonesia	Indonesia
224	Thiti	Snabboon	Chulalongkorn University	Thailand
225	Renan P	Souza	University of Souther Santa Catarina	Italy
226	Sruthi	Srinivasan	University of Waterloo	Canada
227	Steven John	Stack	Wayne State University	USA
228	Maria E	Stefanidou	University of Athens	Greece
229	Tin Tin	Su	University of Malaya Medical Centre	Malaysia
230	Henri	Sueke	Royal Liverpool University Hospital	UK
231	Sarina	Sulong	Universiti Sains Malaysia	Malaysia
232	Syed Hassan	Syed Abdul Aziz	Universiti Sains Malaysia	Malaysia
233	Wojciech	Szczeklik	Jagiellonian University School of Medicine	Poland
234	Samuel	Takongmo	Centre Hopspitalier Universitaire de Yaounde	Cameroon
235	Firouzeh	Talischi	Shahid Beheshti University of Medical Sciences	Iran
236	Florence	Tan	Sarawak General Hospital	Malaysia
237	Andrew, Khee Guan	Tan	Universiti Sains Malaysia	Malaysia
238	Peng Chiong	Tan	University of Malaya Medical Centre	Malaysia
239	Kok Leong	Tan	International Medical University	Malaysia
240	Manish	Taneja	Singapore General Hospital	Singapore
241	Ulf Karl-Martin	Teichgraber	Charite University Hospital	Germany
242	Mysore V	Tejesvi	University of Mysore	India
243	Conjeevaram Rajendrarao	Thambidorai	University of Malaya Medical Centre	Malaysia
244	Chaiwetch	Thanapaisan	Chiangrai Regional Hospital	Thailand
245	John	Tharakan	Universiti Sains Malaysia	Malaysia
246	Sharon	Theophilus	Hospital Sultanah Aminah	Malaysia
247	Bernard	Thong	Tan Tock Seng Hospital	Singapore
248	Satish	Totey	Advanced Neuroscience Allies Private Limited	India
249	Priyanka Pushkarbhai	Trivedi	National Institute of Pharmaceutical Education and Research	India
250	Rupal H	Trivedi	Medical University of South Carolina	USA
251	Nujaimin	Udin	Hospital Kuala Lumpur	Malaysia
252	Otas E	Ukponmwan	Obafemi Awolowo University	Nigeria
253	Guillermo	Valladares	Peruvian-Japanese Medical Center	Peru
254	Esther	van de Vosse	Leiden University Medical Center	The Netherlands
255	Guido	van Hal	University of Antwerp	Belgium
256	Jacques	Van Rooyen	Cape Peninsula University of Technology	South Africa
257	Arturo Z	Vasquez-Parraga	The University of Texas-Pan American	USA
258	Johan	Verhaeghe	Katholieke Universiteit Leuven	Belgium
259	Borlingegowda	Viswanatha	Bangalore Medical College and Research Institute	India
260	Paulo D	Vitória	Universidade da Beira Interior	Portugal
261	Martin	Voracek	University of Vienna	Austria
262	Wan Haslindawani	Wan Mahmood	Universiti Sains Malaysia	Malaysia

No.	First Name	Last Name	Affiliation	Country
263	Jingh	Wang	University of Texas Southwestern Medical Center	USA
264	Michael Anthony	Waugh	Nuffield Hospital	UK
265	Than	Winn	MAHSA University College	Malaysia
266	Scott M	Wright	Johns Hopkins University	USA
267	Tian-Ci	Yang	Xiamen University	China
268	Recep	Yildizhan	Yuzuncu Yil University	Turkey
269	Cheng Har	Yip	University of Malaya Medical Centre	Malaysia
270	Jinlu	Yu	The First Hospital of Jilin University	China
271	Nurasyikin	Yusof	Universiti Kebangsaan Malaysia Medical Centre	Malaysia
272	Ahmad	Zakaria	Universiti Sains Malaysia	Malaysia
273	Sa'ed H	Zyoud	Universiti Sains Malaysia	Malaysia

Contents

Editorial

Interesting Asian Plants: Their Compounds and Effects on Electrophysiology and Behaviour

Jafri Malin Abdullah

Submitted: 1 May 2011
Accepted: 15 Aug 2011

Malaysian Journal of Medical Sciences, Universiti Sains Malaysia Press, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia

Abstract

There have been numerous non-scientific reports on the behavioural effects of Asian plants in humans who consumed these plants wholly or part thereof. Knowledge passed from generation to generation informs us of plants that increase effort and stamina, such as during paddy planting after the ingestion of *Mitragyna speciosa* Korth (ketum) as a tea supplement. *Centella asiatica* and *Myristica fragrans* are used as herbs to improve memory and to treat epilepsy, respectively. *Zizyphus mauritiana* is used to treat headache and burn pain, acts as an antitussive, and reduces rigor mortis immediately after death. These plants, which have been identified to exhibit analgaesic, muscle-relaxing, and nootropic effects, may contain important bio-compounds for medicinal chemistry and pharmaceutical research in Malaysia. The electrophysiology properties of these plants and their effects on epilepsy, behaviour, and pain will lead Malaysia to future new drug discoveries.

Keywords: *behaviour, electrophysiology, GABA, long-term potentiation, medicinal chemistry, neurosciences, plants*

Many Asian plants touted to be useful in the treatment of psychological disorders have been identified to possess psychoactivity properties. A psychoactive plant is one that has phytochemical compound(s) that affect the central nervous system, producing changes in mental activity and/or behaviour (1). These herbal remedies may produce changes in mood and behaviour as well as decrease epileptic attacks. Currently, a fourth of the world's pharmaceutical drugs contain phytochemicals directly extracted from higher plants, or their derivates (2). The electrophysiological properties of 4 plants, namely *Centella asiatica* (pegaga) and its compound (asiatic acid), *Zizyphus mauritiana* (bidara) and its crude extracts, *Myristica fragrans* (Malaysian nutmeg) and its extracts, and *Mitragyna speciosa* Korth (ketum) and its crude extracts, have been studied to look at their in vivo and in vitro effects.

Asiatic acid from the Malaysian *C. asiatica*, at 0.125 µg, was shown to inhibit acetylcholinesterase enzyme. Asiatic acid also produced a depressant effect to the synaptic activity in extracellular recording experiments. The major effects of asiatic acid were the dose- and time-dependent increases in the intensity and duration of gamma-aminobutyric acid $(GABA)_A$ blocker–mediated inhibition compared with $GABA_B$ blocker, which showed no response. This depressant effect was not reversible after a 30-minute washout of the asiatic acid in the recording chamber when exposed to known GABA antagonist bicuculine or phaclofen. These findings were consistent with asiatic acid acting selectively on the $GABA_B$ receptor, which is metabotropic, and not on the $GABA_A$ receptor. The impairment of excitatory synaptic transmission at the Schaffer collateral–CA1 synapse by asiatic acid indicated that this effect was caused by its direct or indirect action on GABAergic receptors. Asiatic acid's interaction with specific $GABA_B$ receptors leads to the receptors' opening that effected the outflow of potassium ions from the neuronal cell and, subsequently, the hyperpolarisation of the neuronal cell, resulting in the decreased excitatory post-synaptic potential (EPSP) within time. The half maximal inhibitory concentration (IC_{50}) of asiatic acid derived from *C. asiatica* was 14 µM(3).

From the dose-dependent inhibitory effects of asiatic acid, the concentration response current were fitted, and further examination of the inhibitory effect of asiatic acid on the field

EPSPs (fEPSPs) evoked in the CA1 subregion was performed. Asiatic acid decreased fEPSPs in a dose-dependent manner, with an IC_{50} of approximately 14 µM(3).

The effects of asiatic acid on active and passive behaviour indicated that it could contribute to better performances in learning and memory via an inhibitory GABAergic effect through $GABA_B$ receptor (4).

The essential oil of *M. fragrans* was tested in *Xenopus* oocytes that were injected with $GABA_A$ receptor comprising of 2α1, 2β2, and γ2s subunits by using automated fast perfusion system during 2-microelectrode voltage-clamp measurement (5). At 1.0% essential oil and 5 uM GABA, the percentage of current stimulation was more than 100%. Two compounds from *M. fragrans*, X1000a and X1000b, also exhibited a significant increase in current flow at 100 uM (Figure 1a and 1b) (6). These findings showed that the compounds acted at the $GABA_A$ receptor as positive modulators or agonists. The compounds also showed interesting antiepileptic effects, as observed in the improvement of the number and the type of seizures in rats monitored with intracranial implants using video wireless telemetry methods (Figures 2 and 3) (6).

Another similar experiment using *Z. mauritania* did not show the potential to generate GABA current even for a small percentage (Figure 1c). The mechanism of action of *Z. mauritania* in pain reduction was unlikely via the GABA pathway, dissimilar to the *M. fragrans* experimental findings (6).

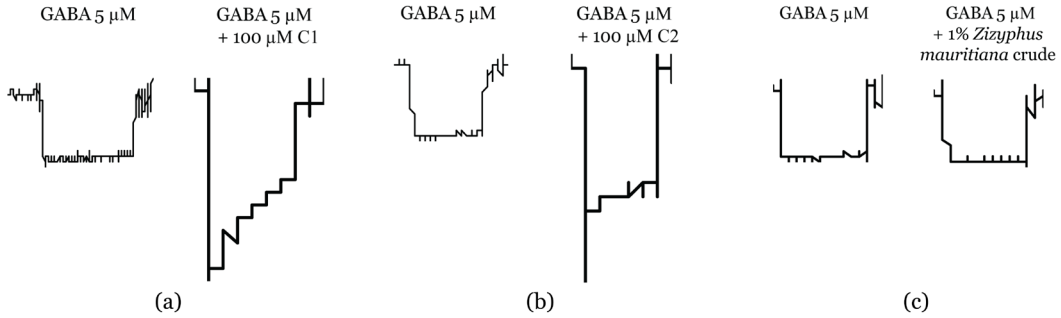


Figure 1: Representative current traces recorded from oocyte expressing $GABA_A$ receptors composed of α1, β2 and γ2 subunits at 100 µM concentrations of X1000a and X1000b. The figure shows the difference of the GABA current stimulation 5 µM GABA and in the presence of (a) 100 µM X1000a, (b) 100 µM X1000b, or (c) 1% *Z. mauritiana* root crude extract.

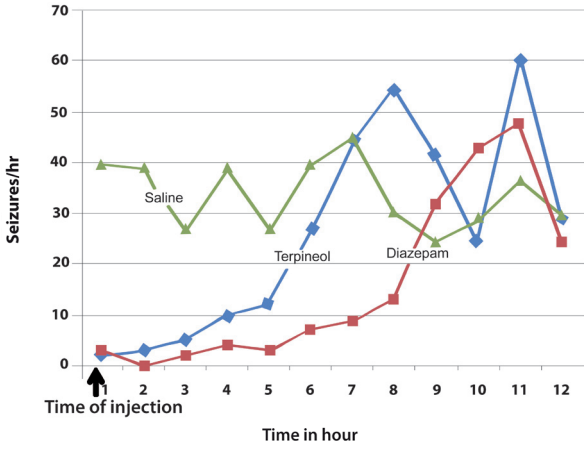


Figure 2: Antiepileptic effects of X1000a (50 mg/kg) on GAERS rat in comparison with diazepam (positive control) and normal saline (negative control).

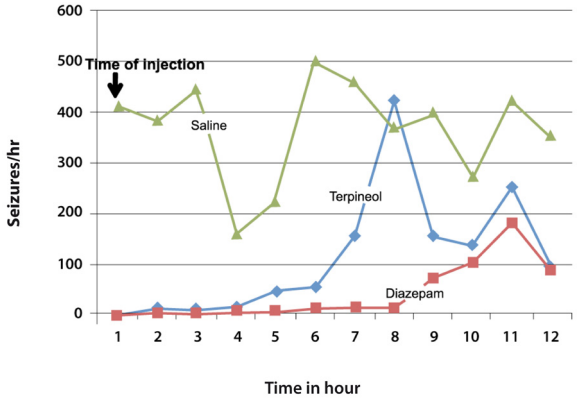


Figure 3: Antiepileptic effects of X1000b (50 mg/kg) on GAERS rat in comparison with diazepam (positive control) and saline as (negative control).

Our experiments with the standardised crude extracts of *M. speciosa* Korth demonstrated that, in the rat hippocampal slices, superfusion of extracts brought about a long-lasting reduction of neurotransmission at the synapses connecting the Schaffer collateral–commissural fibres with CAl hippocampal pyramidal cells. Normalised fEPSP responses from groups treated with 0.0001%, 0.001%, 0.005%, and 0.01% extracts showed irreversible decreases of the fEPSP amplitude for approximately 40 minutes. In order to determine the recovery from the inhibitory effect exerted by *M. speciosa*, the peak amplitude was measured during 20-minute washout. The drug-induced inhibition of the peak amplitude was irreversible during this period (7).

The *M. speciosa* crude extract showed inhibitory and excitatory effects concurrently. This trend is because many compounds are present in the crude extract, all of which have various effects (8); specific compounds eliciting the observed effects could not be differentiated. From the trend, we can also postulate that the response showed continuously decreasing pattern due to the irreversible action of the compounds available. The two highest concentrations of *M. speciosa* (0.05% and 0.1%) showed consistently decreasing patterns from the beginning of the experiment, especially at 0.1% in which a drastic decrease was observed. The high concentration of biocompounds is believed to have contributed to this irreversible effect.

The highest dose of *M. speciosa* Korth standardised methanol extract was necessary for the learning acquisition study using Sprague Dawley rats. However, when administered acutely (single dose), no consolidation occurred, which leads to the failure of information retrieval (memory impairment) in both inhibitory avoidance tasks (one-way passive avoidance and two-way active avoidance tests) without any acute pathological effects to the (Figure 4) (9).

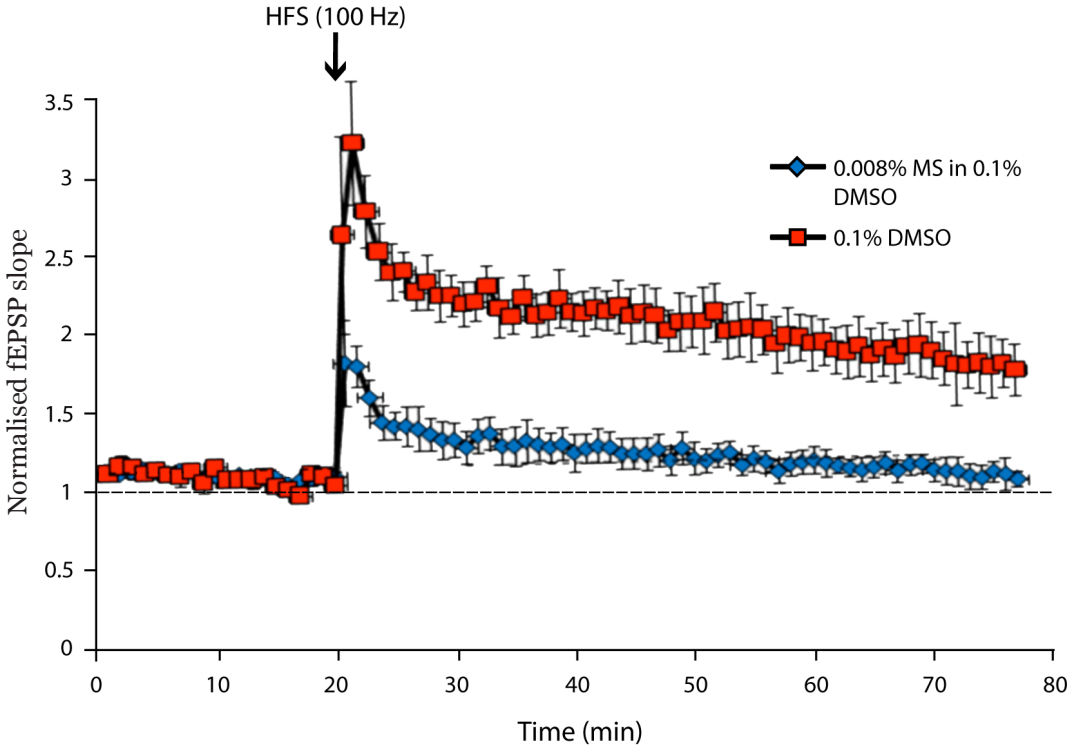


Figure 4: Long-term potentiation (LTP) experiment indicating stable baseline responses were recorded in hippocampal area CA1 of slices from both groups for 20 min. LTP-inducing high frequency stimulation (HFS) consisted of one train of HFS (100 pulses at 100 Hz). Perfusion with vehicle (0.1% dimethyl sulfoxide, DMSO) resulted in rapid and long-lasting potentiation. The group treated with 0.008% *M. speciosa* Korth standardised methanol extract dissolved in 0.1 % DMSO showed inhibition of the induced long-term potentiation, a short-term potentiation. The initial slope of the field excitatory post-synaptic potential (fEPSP) has been normalised to the average baseline value during the perfusion. The points represent means, and the error bars represent SEM (n = 6, respectively).

Across the concentration of *M. speciosa* Korth standardised methanol extract tested in this experiment, the fEPSPs appeared to decrease over time; this effect was irreversible. The IC_{50} of 0.008% extract showed short-term potentiation (STP) response in rat CA1 hippocampus neurons. The induction of STP resulted in less Ca^{2+} influx into post-synaptic cell and triggered lower threshold compared with the long-term potentiation. STP is dependent to N-methyl-D-aspartate receptor, which is required in learning the process.

The multitude of electrophysiological findings from standardised crude extracts or identified plant compounds will eventually help the pharmaceutical industries of Malaysia to decide, after screening, which plants and their compounds are the future drug candidates that can act on the central and peripheral nervous systems.

Acknowledgements

I would like to acknowledge the contributions of Professor Dr Gerhard Rammes of the Max Planck Institute of Psychiatry and the Department of Anaesthesiology, Technical University, Klinikum Rechts der Isar, Munich, Germany; Professor Dr Stefann Hering of the Institute of Pharmacology and Toxicology, University of Vienna, Austria; Hans Reiner Polder of NPI Electronic GmbH, Tamm, Germany; and the Deutscher Akademischer Austausch Dienst–Universiti Sains Malaysia Electrophysiology Summer School 2009 headed by Professor Dr Michael Horner of the Georg-August University, Gottingen. The electrophysiology experiments and animal behaviour and epilepsy studies were done in part under my supervision of these Master students in Neurosciences, Universiti Sains Malaysia: Harizal Senik on *M. speciosa* Korth, Nasir Mat Noor on *C. asiatica*, Ahmad Tarmizi Che Has on *Z. mauritiana* as well as on *M. fragrans*, and post-doctoral fellow Dr M Rafiqul Islam, who worked on the effects of these compounds on epileptic rat models. I also would like to thank my other investigators Professor Dr Sharif Mahsufi Mansor of the Centre for Drug Research, Universiti Sains Malaysia; Associate Professor Dr Hasnah Osman of the School of Chemical Sciences, Universiti Sains Malaysia; and Dr Habsah Mohamad of the Institute of Marine Biotechnology, Universiti Malaysia Terengganu, who had equal contributions to this research.

Correspondence

Professor Dr Jafri Malin Abdullah
FASc (Malaysia), MD (Universiti Sains Malaysia), PhD (University of Ghent), FRCS (Ed), FACS (USA), FAAN (USA), DSC Neurosurgery (Belgium)
Editor-in-Chief
Medical Journal of Medical Sciences
Universiti Sains Malaysia Press
Universiti Sains Malaysia Health Campus
16150 Kubang Kerian
Kelantan, Malaysia
Tel: +609-767 6972
Fax: +609-767 2359
Email: mjms.usm@gmail.com

References

1. Rita S. *Psychoactive medicinal plants: Hallucinogenic and narcotic drugs*. New Delhi (IN): Global Vision Publishing House; 2006.

2. Wijeweera P, Arnason JT, Koszycki D, Merali Z. Evaluation of anxiolytic properties of Gotukola – (*Centella asiatica*) extracts and asiaticoside in rat behavioral models. *Phytomedicine*. 2006; **13(9–10)**:668–676.

3. Nasir MN. The effects of *Centella asiatica* on the psychoactivity of the rat hippocampus: in vivo and In vitro study [master's thesis]. [Kelantan (MY)]: Universiti Sains Malaysia; 2011.

4. Nasir MN, Habsah M, Zamzuri I, Rammes G, Hasnan J, Abdullah J. Effects of asiatic acid on passive and active avoidance task in male Sprague-Dawley rats. *J Ethnopharmacol*. 2011;24;**134(2):**203–209.

5. Baburin I, Beyl S, Hering S. Automated fast perfusion of *Xenopus* oocytes for drug screening. *Pflugers Arch*. 2006;**453(1):**117–123.

6. Ahmad Tarmizi CH. The inhibitory effects of *Zizyphus mauritiana* and *Myristica fragrans* on GABA (A) receptors (α1β2γ2) An in vitro study [master's thesis]. [Kelantan (MY)]: Universiti Sains Malaysia; 2011.

7. Harizal S. Behaviour pharmacology of *Mitragyna speciosa* Korth standardized methanol extract in an animal model [master's thesis]. [Kelantan (MY)]: Universiti Sains Malaysia; 2011.

8. Matsumoto K, Yamamoto, LT, Watanabe K, Yano S, Shan J, Pang PK, et al. Inhibitory effect of mitragynine, an analgesic alkaloid from Thai herbal medicine on neurogenic contraction of the vas deferens. *Life Sci*. 2005;**78(2)**:187–194.

9. Harizal SN, Mansor SM, Hasnan J, Tharakan JK, Abdullah J. Effects of acute administration of *Mitragyna speciosa* Korth standardized methanol extract in animal model of learning and memory. *J Med Plant Res*. Forthcoming.

Review Article

The Importance of Animal Models in Tuberculosis Vaccine Development

Armando Acosta[1], Mohd Nor Norazmi[2], Rogelio Hernandez-Pando[3], Nadine Alvarez[1], Reinier Borrero[1], Juan F Infante[1], Maria E Sarmiento[1]

Submitted: 13 Sep 2011
Accepted: 26 Sep 2011

[1] *Instituto Finlay, PO Box 16017, Cod 11600, La Habana, Cuba*

[2] *School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[3] *National Institute of Medical Sciences and Nutrition Salvador Zubiran, Calle Vasco de Quiroga 15, Tlalpan, CP 14000, Mexico DF, Mexico*

Abstract

Research, development, and production of vaccines are still highly dependent on the use of animal models in the various evaluation steps. Despite this fact, there are strong interests and ongoing efforts to reduce the use of animals in vaccine development. Tuberculosis vaccine development is one important example of the complexities involved in the use of animal models for the production of new vaccines. This review summarises some of the general aspects related with the use of animals in vaccine research and production, as well as achievements and challenges towards the rational use of animals, particularly in the case of tuberculosis vaccine development.

Keywords: *animal models, animal testing alternatives, research and development, tuberculosis, vaccines*

Animal Models in Vaccine Development

Vaccines are instrumental in saving millions of lives every year, contributing to the control of various infectious diseases at the global and regional levels. New vaccines developed with the use of modern technologies could prevent and treat infectious and non-infectious diseases, such as tuberculosis (TB), malaria, human immunodeficiency virus (HIV) infection, hypertension, and diabetes, which are currently without any effective licensed vaccines (1).

With the explosive development in microbiology, immunology, biochemistry, biotechnology, bioinformatics, and other areas of knowledge, vaccinology has become one of the more dynamic areas of biomedical research (1). The development of vaccines for the improvement of existing ones; the search for new vaccines to prevent infectious diseases not previously covered by vaccines, such as acquired immunodeficiency syndrome, TB, and malaria; and the work on therapeutic vaccines for chronic infectious and autoimmune diseases as well as for cancer, allergies, and addiction characterise the current landscape of vaccine research and development at the international level (2).

The use of vaccines has marked differences compared with curative medicines. Vaccination is a health intervention to be used in a large number of healthy people, including newborns and children. Therefore, safety testing is of paramount importance in the development and production of any vaccine (3).

Characterisation of vaccines has additional difficulties compared with other pharmaceuticals due to the complexity of the antigens they contain and the production processes (3). In addition, the production processes include the interaction with multiple agents that can be present in the final lot as preservatives and adjuvants (3).

The main target of vaccination is the elicitation of a protective or therapeutic immune response, which is an intrinsically complex process that have been impossible to reproduce in vitro; therefore, the evaluation of vaccines still needs the use of complex organisms, making the use of animals an unavoidable requisite (4). The introduction of the smallpox and rabies vaccines by Jenner and Pasteur, respectively, are one of the more relevant examples (4).

Animals are used in all the stages of research, development, production, and quality control of vaccines (5). Although there is no exact figure, it is estimated that vaccine research, development, production, and quality control require around 15% the total number of animals used in biomedical research (5). At the research and

development stage, animals are used for adjuvant selection, immunogenicity and safety studies, tests for route and dose of administration as well as formulation, among other aspects (6). In vaccine production, the animal use is only required for selected viral vaccines (5). Batch-release testing is the most demanding aspect in terms of animal use in vaccinology; it is mainly related to studies of toxicity and potency (5,7).

Animal models are of paramount importance in different aspects of vaccinology, such as analyses of the mechanism, route, and transmission of the disease, the host immune response to infection and vaccination, and the duration of induced protection (4,6). The development of novel concepts, such as mucosal, maternal, in utero, neonatal, and elderly vaccination, and the exploration of new vaccine technologies, including delivery systems, are among the important aspects highly dependent on the use of animals in the research stage (4,6,8).

However, in the selection of the animal model, several aspects should be taken into consideration in order to have an appropriate and justified use of the animals, such as issues concerning the host and the pathogenic microorganism (4,6).

In relation to the experimental animal host, important elements are the ontogeny of the immune system; the mucosal immune system; the possibility of the transfer of immune effectors to placenta, colostrum, and milk; the duration of neonatal, adolescent, and adult periods; the receptors involved; and the duration of the immune memory (4,6). In the case of human vaccines, the highest degree of similitude between the human host and the experimental animal species used for the development of the vaccine is of great importance (4,6).

In relation to the pathogens, it is important to intensively research their genetic and antigenic characteristics, virulence, pathogenesis, route of entry, replication, and dissemination (4,6).

As mentioned previously, the use of animals covers the entire spectrum in vaccinology, from basic research up to production and quality control of the final product (4,6,8).

Taking into consideration the large number of animals and the frequency of tests needed in vaccine studies, the development of methods that allow the reduction of animal use in the different aspects of vaccine production is a priority (5). The use of the 3R principles—replacement, reduction, and refinement—in the vaccine industry are the main strategic tool towards the aim to decrease the dependence on animals in vaccinology (5,9).

The production technology of viral vaccines has witnessed a positive evolution pertaining to the use of animals. For poliomyelitis vaccines, the use of primary or subcultured monkey kidney cells has been substituted with the use of continuous cell lines; in the same way, for vaccines against rabies, influenza, and smallpox, the use of continuous cell lines has replaced the use of baby rabbits and mice, embryonated chicken eggs, and calf skin, respectively, resulting in a substantial reduction in animal consumption while maintaining the safety of the products (5).

Once a vaccine product is licensed, a mandatory element is the monitoring of quality and safety through batch-release testing, which evaluate relevant elements such as vaccine safety, potency, and purity. The batch-release testing is a regulatory obligation and demands approximately 80% of all animals used in vaccine production (4,7).

Important advances have been made in the reduction of the use of animals in batch-release testing. In the potency evaluation, a test aimed to determine the potential to induce a protective immune response, the replacement of multiple-dilution by single-dilution assays using antibody determination for diphtheria and tetanus toxoid testing allows the substantial reduction of the use of mice and guinea pigs, thus meeting the principles of refinement and reduction (5,9). Similarly, in rabies vaccines, the use of single dilution in the lethal challenge test allows the reduction in the number of animals used, thus meeting the principle of reduction (5). In general, potency assays based on challenge tests with severe clinical signs are being refined with the use of humane endpoints (5).

In terms of safety testing, in the weight-gain test for whole-cell pertussis vaccine, a reduction of the number of animals has been accepted (5). In the case of the residual toxicity test for diphtheria vaccine, replacement of the use of guinea pigs by the Vero cell test have been approved (5). In the evaluation of neurovirulence of oral polio vaccine, the use of transgenic mouse as a refinement of the assay and the use of polymerase chain reaction as a replacement alternative are under validation (5).

Another important example is the replacement of the pyrogenicity test in rabbits by the limulus amoebocyte lysate assay that have been accepted for several vaccines, such as for hepatitis A, typhoid, yellow fever, influenza, rabies, and *Haemophilus influenzae* type b (5).

The implementation of the 3R principles to vaccine industry is achieving important advances:

18% reduction in the use of animals has been reported despite 20% and 17% increases in the activities of production and research and development, respectively (10).

A critical aspect for the successful introduction of new tests according to the 3R principles in vaccine industry is their acceptance by the regulatory authorities; the validation process, which is aimed to demonstrate the relevance and reliability of the new test in a study involving multiple laboratories, is of paramount importance (5). This process, in general, is long and complex, but the final acceptance by the regulatory authority does not guarantee the automatic implementation as a routine test in all laboratories (5). Several factors are involved in this situation; among them, the lack of harmonisation is the main obstacle because the acceptance by one authority does not mean the acceptance by all the regulatory bodies (5).

Other obstacles for the implementation of the new methods are related to economical problems, difficulties in the implementation, attitude at the laboratory level, pragmatic reasons, and lack of training (5).

Efforts are underway in order to speed the introduction of new tests in vaccine industry meeting the 3R principles. Among the initiatives that can potentially facilitate this task are the implementation of harmonising guidelines, mutual acceptance of data, training courses in 3R methods, the decrease or suppression of fees related to the submission of dossier variations to regulatory authorities, and the introduction of new paradigms in quality control, such as the "consistency approach", which focuses on a set of non-animal test models and gives importance to the in-process testing, the implementation of good manufacturing practice, and the quality assurance, in order to reduce the need of animal use (5,7).

The Case of Tuberculosis Vaccines

One example of the complexities associated with the use of animal models in vaccine development is that of TB (11–13).

TB is one of the most prevalent diseases in developing countries. World Health Organisation estimates that 8.7 million new cases and approximately 1.6 million deaths occur annually (14). The challenge in controlling the transmission of the causative organism *M. tuberculosis* is compounded by the difficulties in diagnosis, the emergence of multi-drug resistant strains, the poor treatment compliance, and the presence of co-infection with HIV (15). The current vaccine against *M. tuberculosis*, *M. bovis* bacille Calmette–Guerin (BCG), has been extensively evaluated, and it is estimated that, currently, more than 3 billion people have received BCG (16). Thus, BCG is the most widely used vaccine in preventing TB especially in childhood (17–22). However, it has also been established that the protection afforded by BCG is highly controversial (17–22). Thus, a more effective TB vaccine is urgently needed.

Projects related with TB vaccine research and development tend to be multicentred and use challenge models with *M. tuberculosis* as protection criterion (23,24). The use of challenge experiments to determine the protective capability of the vaccine candidate has intrinsic complexities due to the slow growth of the microorganism, the length of the experiments (usually between 6-9 months), and the requirement of biosafety level 3 facilities (23,24). Due to the complex nature of the challenge studies, the high specialisation needed, and the expensive facilities required for such studies, this kind of experiments normally are carried out in international reference centres where several vaccine candidates belonging to different research groups are evaluated following standard experimental protocols and evaluation criteria (23,24) (Figures 1 and 2).

Several animal models are used in the evaluation of TB vaccines; the advantages and disadvantages of some of them are summarised in Table 1. There are 3 main animal models for the evaluation of new TB vaccines: mice, guinea pigs, and non-human primates (11,12). The models are used in a sequential way: first, mice, followed by guinea pigs and non-human primates as an optional model. The go/no-go criterion for the change of stage is based on the achievement of a better protection than the one obtained with BCG, the current vaccine in use, or similar protection to BCG but with improved safety (25).

Mouse model

In general, mice are the species of choice for most biomedical research, in particular for immunological evaluation due to their similitude to human biology, which is reflected at the genomic level (11,12).

Despite the similitude in the immune system between human and mouse, there are differences that are relevant in the case of TB vaccine evaluation, such as the good development of bronchus-associated tissue in mice compared with humans, where this kind of tissue is absent (11).

Figure 1: Biological security cabinet. It is a special cabinet with constant airflow from the back to the front and from the bottom to the top, which permit working with highly virulent organisms such as *M. tuberculosis*. In this equipment, mice are infected with virulent microbacteria and are euthanised for organ harvesting for microbiological, immunological, and histopathological evaluations. The biological security cabinet is a part of biosafety level-3 (BSL-3) facilities of the animal house in the National Institute of Medical Sciences, Mexico City.

Figure 2: Microisolator system. This equipment consists of 2 parallel racks with several acrylic cages connected to a closed airflow system. The system permits a constant flow of clean air to infected animals and prevents the exit of bacteria from the cages. It can house 1000 infected animals and poses no infection risk to the personnel. The microisolator system is a part of the BSL-3 facilities of the animal house in the National Institute of Medical Sciences, Mexico City.

Different routes of infection with *M. tuberculosis* used in this model are intravenous, intraperitoneal, intranasal, intratracheal and aerosol (11–13,23). The low-dose aerosol model, which resembles the natural infection in humans, and the intratracheal model are the two more important evaluation platforms for TB vaccines at the international level (11–13,23,25).

In addition to conventional mouse strains, the use of nude and severe combined immunodeficient mice for the evaluation of safety of live TB vaccines and as surrogate of the effect of vaccination in HIV-infected people is another evaluation tool in use (12). The use of knockout mice for different genes to clarify important mechanisms of the immune response to TB is another important advantage offered by this model (11,12).

One of the most important advantages of the murine model for TB vaccine evaluation is the possibility to screen a high number of vaccine candidates at low cost (11,12).

The main disadvantage of the model is the non-exact reproduction of the protection mechanisms in humans (11,12). Mice have natural resistance to the infection and the composition

Table 1: Advantages and disadvantages of animal models in tuberculosis (TB) vaccine research

Model	Advantage	Disadvantage
Mice	• Possibility to screen a high number of vaccine candidates at low cost • Availability of reagents • Availability of nude, severe combined immunodeficient, and several gene knockout strains	• Non-exact reproduction of the immune protective mechanisms in humans
Guinea pig	• Resemblance with TB in humans	• High cost • Lack of suitable reagents
Rabbit	• Resemblance with TB in humans	• High cost • Lack of suitable reagents
Cattle	• Possibility to develop the study in the natural host (*M. bovis*) • Resemblance with TB in humans • Availability of reagents	• Use of *M. bovis* instead of *M. tuberculosis* • High cost
Non-human primate	• Resemblance with TB in humans • Availability of reagents	• High cost • Small sample size

and organisation of the granuloma are different between mice and humans in several aspects. Despite these disadvantages, the mouse model is used for the first screening of vaccine candidates, and the ones giving good protection are advanced to the next stages of evaluation with the use of other animal models (11–13,25).

Guinea pig model

Vaccine candidates are first evaluated in mice, and the best performers are passed for evaluation in guinea pigs. Guinea pigs develop granulomas similar to that of humans, and they are very susceptible to *M. tuberculosis*, with a rapid progression of active disease and an evolution similar to those observed in humans. Therefore, this model is an important tool for the evaluation of vaccines (11,12,24).

The most important disadvantage of this model is the high cost and the limited availability of immunological reagents to evaluate the immune response in this species (11,12).

Rabbit model

Rabbits produce granulomas with caseous centres that are very similar to the human granulomas, and there are a lot of other similarities between the spectrum of manifestations of TB in rabbits and humans (11,12). This model has been used mainly for the evaluation of pathogenesis and new therapies. The use of this model in vaccine evaluation has been limited (11).

Cattle model

The prevention of TB in cattle by vaccination is an important perspective for the disease control in this economically important species and for the elimination of one of the important sources of zoonotic transmission of TB caused by *M. bovis* in man (11,12,26).

Among the advantages of this model are the possibility to study the natural host, its similarity with human disease, and the availability of commercial reagents for this species (11,12,26).

The main disadvantage of the model is the use of *M. bovis* instead of *M. tuberculosis* as well as the high cost of facilities and animals (11,12,26).

Non-human primate model

This model has been used for the evaluation of new TB vaccines (11–13). The evolution of TB infection and disease in monkeys is similar to that in humans (11,12).

The two main species used are rhesus (*Macaca mulatto*) and cynomolgus (*Macaca fascicularis*) macaques (11). Rhesus macaques are very susceptible to TB, whereas cynomolgus macaques are more resistant. The latter species is efficiently protected by BCG, which makes it suitable for evaluation of new subunit vaccines (11).

Advantages of this model are the resemblance with the evolution of TB in humans, and the availability of reagents for immune evaluation (11,12).

The main disadvantage of the model is its high cost and hence the limitation in using a large number of animals, which interferes with the statistical validation of results (11,12).

The use of this model is restricted to the last part of the pre-clinical evaluation after obtaining solid results in the mouse and guinea pig models (11–13).

Other models

Zebrafish, deer, and other species are also used for the evaluation of new TB vaccines, but their use is restricted to some specific experimental scenarios. However, these models are not considered mandatory in the pre-clinical evaluation of new TB vaccines (11,12).

Towards the Optimisation of Animal Use in Tuberculosis Vaccine Development

As mentioned in the previous sections, multiple approaches directed to the optimal use of animals in vaccine research, development, and production that meet the 3R principles can be applied to replace production processes demanding animals for the production of vaccines. These approaches include the use of cell lines, the refinement, reduction, and replacement of animals in batch release testing of vaccines, and the search for protection correlates (4,5,7,9,10,11,27–33)

The development of genomics, proteomics, and bioinformatics is having a growing impact on the rational design of vaccine candidates, which will result in the reduction of animal use in vaccine research (34).

The strategy of "reverse vaccinology" with different variants and the development of computer programmes for the selection and design of vaccine candidates promise to speed the rational design of efficacious vaccines, saving time and resources and, in particular, allowing a more rational use of animals (34–39).

The availability of the genome sequences of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, mouse, and human, together with the development of multiple computer algorithms, allows the increasing use of in silico bioinformatics methods for the search of new vaccine candidates against TB (40,41).

The use of these methods allows the more rational use of animals, selecting only highly promising candidates for animal immunisation in contrast to conventional methods that requires the in vivo evaluation of a large number of candidates in a trial-and-error fashion (34,35).

Our group is applying this kind of methods in order to identify TB vaccine candidates for the in vivo evaluation, meeting several pre-conditions such as the in vivo overexpression in all the stages of the infection process, the presence of B and T promiscuous epitopes, and the high population coverage in terms of the presentation of the human leucocyte antigen alleles in different populations (42).

Using this method, we have identified several epitopes used for expression in BCG, which demonstrated good profile of immunogenicity and protection in the mice model (19,43, Norazmi et al., unpublished data). The same method has been applied in the evaluation of the potential of new vaccine candidates before going to in vivo studies, as has been the case of proteoliposomes obtained from *M. smegmatis* (42). After the bioinformatics study, the immunogenicity and induction of cross-reactive responses in mice against antigens of M. tuberculosis have been confirmed, validating the results of the bioinformatics study (44).

Conclusion

Research, development, and production of vaccines is still highly dependent of the use of experimental animals. Despite this fact, there is growing interests in the reduction of animals use in the vaccine industry. Many examples of the achievements in the reduction of animal use are available, but many challenges and obstacles still remain. TB vaccine research and development is a relevant example to demonstrate the complexities associated with the use of animals in vaccinology and the efforts to make a more rational use of animal models for the development of new vaccines.

Acknowledgements

Parts of the work described in this review were supported by grants provided by the Ministry of Science, Technology & Innovation (304.PPSK.6150109.K105) and the Ministry of Higher Education (203.PSK.670002), Malaysia.

Authors' Contributions

Conception and design, drafting, critical revision, and final approval of the article: AA, MNM, RHP, NA, RB, JFI, MES

Correspondence

Professor Dr Armando Acosta
MD, PhD (Instituto Finlay)
Calle 17 esquina 198
Rpto. Siboney, Playa
Ciudad de la Habana, Cuba
Tel: +53(7) 271-6911 ext. 106
Fax: +53(7) 208-6075
Email: aracosta2005@yahoo.es

References

1. Levine OS, Bloom DE, Cherian T, de Quadros C, Sow S, Wecker J, et al. The future of immunisation policy, implementation, and financing. *Lancet*. 2011;**378(9789)**:439–448.

2. Schunk MK, Macallum GE. Applications and optimization of immunization procedures. *ILAR J*. 2005;**46(3)**:241–257.

3. Kanesa-thasan N, Shaw A, Stoddard JJ, Vernon TM. Ensuring the optimal safety of licensed vaccines: A perspective of the vaccine research, development, and manufacturing companies. *Pediatrics*. 2011; **127 Suppl 1**:S16–S22.

4. Gerdts V, van Drunen Littel-van den Hurk S, Griebel PJ, Babiuk LA. Use of animal models in the development of human vaccines future microbiology. *Future Microbiol*. 2007;**2(6)**:667–675.

5. Hendriksen C. Three Rs achievements in vaccinology. AATEX [Internet]. 2008 [cited 2011 Sep 5] ; 14 Spec Issue;575–579. Available from: http://altweb.jhsph.edu/bin/q/t/paper575.pdf.

6. Griffin JF. A strategic approach to vaccine development: Animal models, monitoring vaccine efficacy, formulation and delivery. *Adv Drug Deliv Rev*. 2002;**54(6)**:851–861.

7. Hendriksen C, Arciniega JL, Bruckner L, Chevalier M, Coppens E, Descamps J, et al. The consistency approach for the quality control of vaccines. *Biologicals*. 2008;**36(1)**:73–77.

8. Davis HL. Novel vaccines and adjuvant systems: The utility of animal models for predicting immunogenicity in humans. *Human Vaccin*. 2008;**4(3)**:246–250.

9. Metz B, Hendriksen CF, Jiskoot W, Kersten GF. Reduction of animal use in human vaccine quality control: Opportunities and problems. *Vaccine*. 2002;**20(19–20)**:2411–2430.

10. European Vaccine Manufacturers. Animal welfare and vaccine development [Internet]. Brussel (BE): European Vaccine Manufacturers; 2006 [cited 2011 Sep 5]. Available from: http://www.evm-vaccines.org/pdfs/vaccines_and_animal_welfare_fin.pdf.

11. Hernandez-Pando R, Aguilar D, Hernandez R. The contribution of diverse animal models in the evaluation of new vaccines against tuberculosis. In: Norazmi MN, Acosta A, Sarmiento ME, editors. *The art & science of tuberculosis vaccine development* [Internet]. 1st ed. Selangor (MY): Oxford University Press; 2010 [cited 2011 Sep 5]. p. 229–345. Available from: http://tbvaccines.usm.my/.

12. McShane H, Williams A. Preclinical evaluation of tuberculosis vaccines. In: Norazmi MN, Acosta A, Sarmiento ME, editors. *The art & science of tuberculosis vaccine development* [Internet]. 1st ed. Selangor (MY): Oxford University Press; 2010 [cited 2011 Sep 5]. p. 349–373. Available from: http://tbvaccines.usm.my/.

13. Walker KB. Preclinical and clinical development of new tuberculosis vaccines: Regulatory requirements and the transition to phase I and beyond. In: Norazmi MN, Acosta A, Sarmiento ME, editors. *The art & science of tuberculosis vaccine development* [Internet]. 1st ed. Selangor (MY): Oxford University Press; 2010 [cited 2011 Sep 5]. p. 503–523. Available from URL: http://tbvaccines.usm.my/.

14. Young DB, Perkins MD, Duncan K, Barry CE 3rd. Confronting the scientific obstacles to global control of tuberculosis. *J Clin Invest*. 2008;**118(4)**: 1255–1265.

15. Dye C. Tuberculosis 2000–2010: Control, but not elimination. *Int J Tuberc Lung Dis*. 2000; **4(12 Suppl 2)**:S146–S152.

16. Al-Kassimi FA, al-Hajjaj MS, al-Orainey IO, Bamgboye EA. Does the protective effect of neonatal BCG correlate with vaccine-induced tuberculin reaction? *Am J Respir Crit Care Med*. 1995; **152(5 Pt 1)**:1575–1578.

17. Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: Meta-analyses of the published literature. *Pediatrics*. 1995; **96(1 Pt 1)**:29–35.

18. Lanckriet C, Levy-Bruhl D, Bingono E, Siopathis RM, Guerin N. Efficacy of BCG vaccination of the newborn: Evaluation by a follow-up study of contacts in Bangui. *Int J Epidemiol*. 1995;**24(5)**:1042–1049.

19. Norazmi MN, Sarmiento ME, Acosta A. Recent advances in tuberculosis vaccine development**.** *Curr Resp Med Rev*. 2005;**1(2)**:109–116.

20. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA*. 1994;**271(9)**:698–702.

21. Fine PE. Variation in protection by BCG: Implications of and for heterologous immunity. *Lancet*. 1995;**346(8986)**:1339–1345.

22. Brewer TF. Preventing tuberculosis with bacillus Calmette-Guerin vaccine: A meta-analysis of the literature. *Clin Infect Dis*. 2000;**31 Suppl 3**: S64–S67.

23. Izzo A, Brandt L, Lasco T, Kipnis AP, Orme I. NIH pre-clinical screening program: Overview and current status. *Tuberculosis (Edinb)*. 2005;**85(1–2)**:25–28.

24. Williams A, Hatch GJ, Clark SO, Gooch KE, Hatch KA, Hall GA, et al. Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis. *Tuberculosis (Edinb)*. 2005;**85(1–2)**:29–38.

25. Orme IM. The use of animal models to guide rational vaccine design. *Microbes Infect*. 2005;**7(5–6)**: 905–910.

26. Vordermeier M. Development of cattle tuberculosis vaccines. In: Norazmi MN, Acosta A, Sarmiento ME, editors. *The art & science of tuberculosis vaccine development* [Internet]. 1st ed. Selangor (MY): Oxford University Press; 2010 [cited 2011 Sep 5]. p. 405–424. Available from: http://tbvaccines.usm.my/.

27. Amanna IJ, Messaoudi I, Slifka MK. Protective immunity following vaccination: How is it defined? *Hum Vaccin*. 2008;**4(4)**:316–319.

28. McVey DS, Galvin JE, Olson SC. A review of the effectiveness of vaccine potency control testing. *Int J Parasitol*. 2003;**33(5–6)**:507–516.

29. Higbee RG, Byers AM, Dhir V, Drake D, Fahlenkamp HG, Gangur J, et al. An immunologic model for rapid vaccine assessment—A clinical trial in a test tube. *Altern Lab Anim*. 2009;**37 Suppl 1**:19–27.

30. Curlin G, Landry S, Bernstein J, Gorman RL, Mulach B, Hackett CJ, et al. Integrating safety and efficacy evaluation throughout vaccine research and development. *Pediatrics*. 2011;**127 Suppl 1**:S9–S15.

31. De Mattia F, Chapsal JM, Descamps J, Halder M, Jarrett N, Kross I, et al. The consistency approach for quality control of vaccines—A strategy to improve quality control and implement 3Rs. *Biologicals*. 2011;**39(1)**:59–65.

32. Jennings M, Morton DB, Charton E, Cooper J, Hendriksen C, Martin S, et al. Application of the Three Rs to challenge assays used in vaccine testing: Tenth report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Biologicals*. 2010;**38(6)**:684–695.

33. De Boo J, Hendriksen C. Reduction strategies in animal research: A review of scientific approaches at the intra-experimental, supra-experimental and extra-experimental levels. *Altern Lab Anim*. 2005;**33(4)**:369–377.

34. Rinaudo CD, Telford JL, Rappuoli R, Seib KL. Vaccinology in the genome era. *J Clin Invest*. 2009;**119(9)**:2515–2525.

35. Poland GA, Oberg AL. Vaccinomics and bioinformatics: Accelerants for the next golden age of vaccinology. *Vaccine*. 2010;**28(20)**:3509–3510.

36. Hernandez YL, Corona DY, Rodriguez SS, Infante Bourzac JF, Sarmiento ME, Arzuaga NO, et al. Immunization of mice with *Mycobacterium tuberculosis* genomic expression library results in lower bacterial load in lungs after challenge with BCG. *Tuberculosis*. 2006;**86(3–4)**:247–254.

37. Yero D, Pajon R, Perez Y, Farinas M, Cobas K, Diaz D, et al. Identification by genomic immunization of a pool of DNA vaccine candidates that confer protective immunity in mice against *Neisseria meningitidis* serogroup B. *Vaccine*. 2007;**25(28)**:5175–5188.

38. Yero D, Pajon R, Caballero E, Gonzalez S, Cobas K, Farinas M, et al. A novel method to screen genomic libraries that combines genomic immunization with the prime-boost strategy. *FEMS Immunol Med Microbiol*. 2007;**50(3)**:430–433.

39. Amin N, Aguilar A, Chamacho F, Vazquez Y, Pupo M, Ramirez JC, et al. Identification of dengue-specific B-cell epitopes by phage-display random peptide library. *Malaysian J Med Sci*. 2009;**16(4)**:4–14.

40. Tang ST, van Meijgaarden KE, Caccamo N, Guggino G, Klein MR, van Weeren P, et al. Genome-based in silico identification of new *Mycobacterium tuberculosis* antigens activating polyfunctional $CD8^+$ T cells in human tuberculosis. *J Immunol*. 2011;**186(2)**: 1068–1080.

41. Wang J, Zhang H, Wang H. Analysis of predicted $CD8^+$ T cell epitopes from proteins encoded by the specific RD regions of *Mycobacterium tuberculosis* for vaccine development and specific diagnosis. *Mol Biol Rep*. 2010;**37(4)**:1793–1799.

42. Nguyen Thi LT, Borrero R, Fernandez S, Reyes G, Perez JL, Reyes F, et al. Evaluation of the potential of *Mycobacterium smegmatis* as vaccine candidate against tuberculosis by in silico and in vivo studies. *VacciMonitor*. 2010;**19(1)**:20–26.

43. Cataldi A, Bigi F, Norazmi MN, Sarmiento ME, Acosta A. Strategies for new generation vaccine development. In: Norazmi MN, Acosta A, Sarmiento ME, editors. *The art & science of tuberculosis vaccine development* [Internet]. 1st ed. Selangor (MY): Oxford University Press; 2010 [cited 2011 Sep 5]. p. 186–208. Available from: http://tbvaccines.usm.my/.

44. Rodriguez L, Tirado Y, Reyes F, Puig A, Kadir R, Borrero R, et al. Proteoliposomes from *Mycobacterium smegmatis* induce immune cross-reactivity against *Mycobacterium tuberculosis* antigens in mice. *Vaccine*. 2011;**29(37)**:6236–6241.

Original Article

The In Vitro Antimicrobial Activities of Metabolites from *Lactobacillus* Strains on *Candida* Species Implicated in *Candida* Vaginitis

Adenike A O Ogunshe[1], Mopelola A Omotoso[2], Victoria B Bello[3]

Submitted: 21 Jan 2011
Accepted: 14 Apr 2011

[1] *Applied Microbiology and Infectious Diseases Unit, Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Oyo State 900001, Nigeria*

[2] *Department of Chemistry, Faculty of Science, University of Ibadan, Ibadan, Oyo State 900001, Nigeria*

[3] *Microbiology and Virology Unit, Laboratory Technology Training School, Faculty of Science, University of Ibadan, Ibadan, Oyo State 900001, Nigeria*

Abstract

***Background*: Research from developing countries, such as Nigeria, on *Lactobacillus* species in the female urogenital tract and their role as a barrier to vaginal infection is limited. Therefore, the aim of this study was to assess the clinical biotherapeutic potential of indigenous *Lactobacillus* species.**

***Methods:* Antimicrobial metabolites production were characterised using simple and easily reproducible qualitative and quantitative methods. The in vitro inhibitory effect of *Lactobacillus* antimicrobials on vulvovaginal candidiasis–associated *Candida* species was investigated using modified agar spot and agar well-diffusion methods.**

***Results:* The maximum levels of lactic acid, hydrogen peroxide, and diacetyl from 20 vaginal *Lactobacillus* strains from diseased subjects were 1.46 mg/L, 1.36 mmol/L, and 1.72 mg/L respectively. From the 4 healthy subjects, the maximum level of lactic acid was 1.08 mg/L; hydrogen peroxide, 1.36 mmol/L; and diacetyl, 0.86 mg/L. The maximum productions of these substances occurred between 72 and 120 hours of incubation. The in vitro antagonistic activities of vaginal *L. acidophilus*, *L. fermentum*, *L. brevis*, *L. plantarum*, *L. casei*, *L. delbrueckii*, and *L. jensenii* from diseased subjects inhibited a maximum of 5.71% of the 35 *Candida* species tested, while vaginal *L. acidophilus* and *L. plantarum* from healthy subjects inhibited between 57.1% and 68.6% of *Candida* species in vitro.**

***Conclusion:* Antimicrobial-producing lactobacilli can be considered as adjunct biotherapeutic candidates for the treatment of vulvovaginal candidiasis.**

Keywords: *antifungal agents, antimicrobial agents,* Candida*, contraceptives,* Lactobacillus*, vulvovaginal candidiasis*

Introduction

The vaginal microflora in its totality is a flexible population that occupies a particular ecological niche and acts as a barrier to the establishment of other microorganisms. Additionally, it has been reported that the vaginal ecosystem contains microbiota that protect it from invading pathogens, including those that are sexually transmissible and those that cause urinary tract infections (1,2). However, the degree of acute stress tolerated by the vaginal microflora must have defined limits. If the acute stress is too extreme, the original microflora may collapse and therefore be unable to provide protection against pathogenic microorganisms that can rapidly proliferate in the environment (3,4), resulting in genitourinary diseases.

Sexually transmissible diseases (STDs) are some of the least recognised health problems worldwide, especially in developing countries like Nigeria (5). In spite of advances in diagnosis and treatment, the number of STD cases has continued to rise and has reached epidemic proportion in many countries. Candidiasis is a mycotic human infection caused by *Candida albicans* and other related pathogenic *Candida* species, and it is one of the most common STDs.

Approximately 3 million or about 75% of women experience vulvovaginal candidiasis (VVC), and almost 10% of them have recurrent VVC (6–8). *Candida* spp. are reported as the most commonly cultured pathogenic microorganisms, and vaginal candidiasis is one of the most common infections seen in general practice (9).

Studies have suggested that the candidal problem is not under control and, in fact, is worsening. Although *Candida* infections are usually treated with an array of antimycotic agents such as azoles, polyenes, echinocandins, allylamines, and other derivatives, the emergence of antimycotic-resistant candidal pathogens, especially the potential widespread dissemination of resistance, has become a major public health concern (10–12). The prevalence of VVC or *Candida* vulvovaginitis is therefore expected to increase. Antifungal resistance has been reported in most *Candida* species, which are the aetiological agents of VVC (5), even in addition to other adverse effects of the drugs. Thus, there is a need for alternative or adjunct bio-antimycotic means of controlling pathogenic *Candida* species that infect humans.

Lactobacilli, which are well known for their potential ability to prevent diseases in humans (13), are also the predominant members of the vaginal flora in healthy women (14–16). Some researchers from developed countries have reported significant in vitro inhibition of pathogenic vaginal *Candida* by certain lactobacilli species isolated from vaginal and non-vaginal sources (1,2,17,18), but similar studies and information from developing countries, such as Nigeria, are very limited. Therefore, the aim of this study was to investigate the in vitro inhibitory effects of *Lactobacillus* antimicrobials from the vaginas of healthy and diseased Nigerian females on *Candida* species associated with human vaginal candidiasis. A comparison of the inhibition profiles of *Candida* species by the antimicrobial metabolites produced by vaginal lactobacilli from healthy and diseased subjects with that of commercial antifungals and contraceptives was also conducted.

Materials and Methods

Strains and culture conditions

The *Candida* strains used were obtained from the original stock of the microbial collections at the Department of Medical Microbiology & Parasitology, University College Hospital, Ibadan, Nigeria. High vaginal swabs and/or endocervical swabs were obtained from female patients aged 3–62 years old who presented at the special treatment clinic of University College Hospital, Ibadan, Nigeria (6). Initial isolation of bacteria from the vaginal specimens were performed on blood agar, chocolate agar, Sabouraud dextrose agar (SDA; LAB M, UK), cysteine lactose electrolyte deficient agar (LAB M, UK), and Chrom agar that were incubated at 32 °C. The *Lactobacillus* strains were obtained from some of the clinical specimens from diseased patients. Control samples were obtained from a control group of 4 healthy subjects, who were 29–35 years of age, had had 4 previous regular menstrual flows, and who were not on antibiotic or antifungal therapy 6 to 12 months prior to collection of the specimens.

The *Lactobacillus* strains were cultured on deMann, Rogosa, and Sharpe (MRS) agar (LAB M, UK) and incubated at 35 °C with 5%–10% CO_2. The purity of the strains was checked, and the pure cultures of the *Lactobacillus* strains were phenotypically identified by classical tests including analysis of cell morphology, homo/heterofermentative and biochemical characteristics, sugar fermentation patterns, and growth at different temperatures. The strains were examined microscopically, and the initial confirmation and identification of the lactobacilli was based on Gram's reaction, catalase reaction using hydrogen peroxide (H_2O_2), growth at 15 °C and 45 °C in MRS medium, gas and acid production from glucose fermentation, and fermentation of lactose, sucrose, arabinose, fructose, and mannitol. The isolates that met the preliminary identification criteria were grown in replicates overnight (18–24 hours) in 10 mL Rogosa broth at 35 °C until the weight of the cell mass was 0.05–0.10 g. Additional taxonomic studies were carried out on the purified isolates based on their biochemical and physiological characteristics (15,19,20). The cells were centrifuged, washed twice in sterile 0.9% NaCl solution, and were stored at 4 °C in Hogness freezing buffer (3.6 mM K_2HPO_4, 1.3 mM KH_2PO_4, 2.0 mM Na-citrate, 1.0 mM $MgSO_4$, 12% glycerol); the cells were then kept frozen. When needed, the frozen *Lactobacillus* strains were allowed to thaw at ambient temperature and then reactivated in MRS broth before sub-culturing on MRS agar. Confirmed pure cultures were sub-cultured in MRS broth until active cultures were obtained for further studies.

Pure *Candida* cultures were identified using a combination of colony morphology on culture media, microscopic morphology, and biochemical characteristics including assimilation of the sugars

cellobiose, dextrose, dulcitol, fructose, galactose, glucose, inositol, lactose, maltose, mannitol, mellibiose, raffinose, rhamnose, saccharose, sorbitol, sucrose, and xylose. In addition, fresh wet mount examinations (wet preparations) and germinal tube assays were also performed on the strains, and the final identification was made according to the methods of Kreger-van Rij (21). Pure cultures of the *Candida* strains were stored as *Candida* stock strains at 12 °C on SDA slants containing 0.25 mg streptomycin. The strains were later reactivated in SDA broth and then subcultured by streaking on SDA agar to obtain viable, pure, fresh cultures.

Qualitative determination of antimicrobial metabolites produced by the Lactobacillus *strains using antimicrobial assay*

The detection of antagonistic activity by the *Lactobacillus* strains was performed by the modification of agar spot and agar well-diffusion methods (22) of Tagg et al. (23). Wells of 6.0-mm diameter were bored into sterile SDA plates, and 500 µL of each *Candida* strain was seeded on the sterile agar plates. The plates were then left at ambient temperature under aseptic condition for 30 minutes before 250–1000 µL of each 24- to 36-hour-old *Lactobacillus* strain, in MRS semi-solid agar (5% agar), was added to the agar wells and then directly onto another set of pre-seeded SDA agar plates, as indicated in the agar spot diffusion protocol. The plates were incubated at 35 °C for 24–48 hours, after which the zones of inhibition were measured and the diameter (in mm) was recorded. A zone of inhibition of less than 10.0 mm or an absence of a zone of inhibition were recorded as resistant (negative).

In vitro antimycotic susceptibility testing using antifungal agents

The in vitro susceptibility/resistance of *Candida* strains to antimycotic agents commonly available in Nigeria, namely Diflucan capsules, doxycycline capsules, Fungoral tablets, Mycoten tablets/cream, Canesten tablets/cream (clotrimazole), Tetradox (doxycycline), Mycostatin (nystatin), and Flagyl, was determined using SDA agar plates after 24 and 48 hours of incubation at 35 °C as per the modified agar well-diffusion method (22) of Tagg et al. (23). Wells of 6.0-mm diameter were bored into sterile SDA plates and seeded with 500 µL of each *Candida* strain. The plates were incubated at ambient temperature under aseptic condition for 30 minutes, and afterwards 1 mL of each of the antifungal agents, dissolved in plain, sterile semi-solid agar (5%) at 45 °C, was dispensed into the wells. The plates were incubated uninverted at 35 °C for 24–48 hours, after which the diameters (in mm) of the zones of inhibition were measured and recorded. A zone of inhibition less than 10.0 mm or the absence of a zone of inhibition was recorded as resistant (negative). The concentrations of the *Candida* isolates in the inoculum suspensions used in the test were between 1.6 and 2.4 × 10^3 cells/mL.

In vitro antimycotic susceptibility testing using contraceptives

The modified agar well diffusion method (22) of Tagg et al. (23) was used to determine the in vitro susceptibility/resistance of *Candida* strains to commonly available contraceptives in Nigeria: Confidence (Duofem) tablets (ferrous fumarate, brown), Confidence (Duofem) tablets (ferrous fumarate, white), Norquest Fe tablets (norethindrone/ethinyl oestradiol/ferrous fumarate, green), Norquest Fe tablets (norethindrone/ethinyl oestradiol/ferrous fumarate, yellow), and Ovrette tablets (norgestrel). Wells of 6.0-mm diameter were bored into sterile SDA plates and then seeded with 500 µL of each *Candida* strain. The plates were incubated at ambient temperature under aseptic condition for 30 minutes and then 1 mL of each of the contraceptives, dissolved in plain semi-solid agar (5% agar), was dispensed into the wells. The plates were incubated uninverted at 35 °C for 24–48 hours, after which, the zones of inhibition were measured, and the diameters (in mm) were recorded. A zone of inhibition less than 10.0 mm in diameter or the absence of a zone of inhibition was recorded as resistant (negative). The concentrations of the *Candida* inoculum suspensions used in the test were between 1.6 and 2.4 × 10^3 cells/mL.

Quantitative production of antimicrobial metabolites by lactobacilli species

The stock *Lactobacillus* strains were grown in 10-mL Rogosa broth at 35 °C for 48 hours. When the weight of the cell mass reached between 0.05–0.10 g, 10 mL of each fresh *Lactobacillus* culture was re-suspended in MRS broth and incubated for 24–20 hours at 35 °C without agitation. The culture medium was sampled every 24 hours and centrifuged at 3000 rpm for 15 minutes; the supernatants were then used to test for antimicrobial metabolite production.

Quantitative determination of lactic acid

Production of lactic acid by the *Lactobacillus* strains was determined by the Association of Analytical Communities (AOAC)'s method (24). The supernatant from the MRS broth culture of each test organism was titrated with freshly prepared 0.25 M NaOH and 1 mL of phenolphthalein indicator (0.5% w/v in 50% ethanol). The titratable acidity was calculated as percentage of lactic acid using the following equation:

$$\text{Titratable acidity} = \frac{V_{NaOH} \times N_{NaOH} \times ME}{V_{sample}} \times 100$$

where V = volume (mL), N = normality, and ME = equivalence factor. Each millilitre of 1 N NaOH is equivalent to 90.08 mg of lactic acid.

Quantitative determination of H_2O_2

The H_2O_2 produced by the *Lactobacillus* strains was determined according to the AOAC's method (24). First, 25 mL of supernatant from the MRS broth culture of each test organism was transferred to a 150-mL conical flask. Next, 25 mL of freshly prepared sulphuric acid (H_2SO_4) was added, followed by titration with 0.1 N potassium permanganate ($KMnO_4$). Decolourisation of the sample was regarded as the end point. The H_2O_2 production was calculated as follows:

$$H_2O_2 \text{ production} = \frac{V_{KMnO4} \times N_{KMnO4} \times ME}{V_{H2SO4} \times V_{sample}} \times 100$$

where V = volume (mL), N = normality, and ME = equivalence factor. Each millilitre of 0.1 N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2.

Quantitative determination of diacetyl production

To determine the concentration of diacetyl produced by the *Lactobacillus* strains, we used the method from the Food Chemicals Codex (25). Firstly, 25 mL of the supernatant from the MRS broth culture of each test organism was transferred into a 150-mL conical flask, and 7.5 mL of freshly prepared 1 M hydroxylamine solution was added. The same quantity of hydroxylamine solution was dispensed into another 150-mL conical flask for residual titration. Three drops of bromophenol blue indicator was added to each flask. Both flasks were then titrated with 0.1 N HCl to a greenish-yellow end point. The diacetyl concentration was calculated using the following formula:

$$\text{Diacetyl production} = \frac{(V_a - V_s) \times ME}{V_w} \times 100$$

where V_a = volume (mL) of 0.1 N HCl consumed during the titration, V_s = volume (mL) of 0.1 N HCl consumed in the residual titration, ME = equivalence factor, and V_w = volume (mL) of broth culture used during the titration. The equivalence factor of HCl to diacetyl is 21.52 mg.

Results

Out of the 20 *Lactobacillus* strains that were isolated from the endocervical and high vaginal swabs of diseased patients, 5 were identified as *L. acidophilus* (VL1, VL2, VL3, VL4, VL5), 5 as *L. fermentum* (VL6, VL7, VL8, VL9, VL10), 3 as L. brevis (VL11, VL12, VL13), 2 as *L. plantarum* (VL14, VL15), 2 as *L. casei* (VL16, VL17), 2 as *L. delbrueckii* (VL18, VL19), and 1 as *L. jensenii* (VL20). Out of the 4 strains isolated from the healthy subjects, 2 were identified as *L. acidophilus* (VLHS1, VLHS2) and the other 2 as *L. plantarum* (VLHS3, VLHS4).

As shown in Table 1, the in vitro antagonistic activity of the cell-free supernatant of cultures from the diseased patients' vaginal *Lactobacillus* strains gave an overall maximum inhibition rate of 5.71% against *Candida* species. The zones of inhibition were between minimal (10.0–18.0 mm in diameter) and moderate (20.0–25.0 mm in diameter) susceptibility. However, the healthy subjects' vaginal *Lactobacillus* strains had in vitro inhibition rates of between 57.1% and 68.6% against the *Candida* species (Table 2). The recorded zones of inhibition were between 10.0 and 28.0 mm in diameter, but most of the inhibitory activity was also between minimal and moderate susceptibility. The inhibitory effects of the tested antifungals were more prominent, with inhibition rates ranging 57.7%–92.3% (Table 3). Similarly, most of the inhibitory activities (10.0–35.0 mm in diameter) were either moderate or maximal. No inhibition of the *Candida* strains by the contraceptives was recorded.

There was no consistent pattern in the production of antimicrobial metabolites (lactic acid, hydrogen peroxide, and diacetyl) by the vaginal *Lactobacillus* strains from diseased patients. The maximum antimicrobial metabolite production was between 72 and 120 hours of incubation (Figures 1–3). Similar values of antimicrobial production were also recorded for the vaginal *Lactobacillus* strains from

Table 1: The antimicrobial activities of the cell-free supernatants from the vaginal Lactobacillus strains of diseased patients on the Candida strains

Candida strain	Antimicrobial-producing *Lactobacillus* strain [a]						
	VL2	VL7	VL10	VL14	VL16	VL19	Others
C. albicans							
- 6C1	R	R	R	R	R	20.0	R
- AC1, AC2, BC1,FC1, FC2, GC1, GC2, X1C, IC,2C2, 4C2, H2C	R	R	R	R	R	R	R
C. glabrata							
- BC1, 1C2, X7C, 4C1, 1TC, HC	R	R	R	R	R	R	R
C. pseudotropicalis							
- 2C1	R	R	19.0	R	R	15.0	R
- 6C2	R	25.0	R	R	20.0	R	R
- 2C2B, X7C, 9C2	R	R	R	R	R	R	R
C. tropicalis							
- 9C	10.0	18.0	R	R	R	R	R
- 10C	R	R	R	20.0	R	R	R
- HC, 2TC, 6C, ITC2, 2TC2, 6C1A, HC3, HC1, 9CB	R	R	R	R	R	R	R

R indicates absence of zone of inhibition (resistance).

[a] VL1–5 = *L. acidophilus*, VL6–10 = *L. fermentum*, VL11–13 = *L. brevis*, VL14–15 = *L. plantarum*, VL16–17 = *L. casei*, VL18–19 = *L. delbrueckii*, VL20 = *L. jensenii*.

healthy subjects (VLHS1 = *L. acidophilus 1*, VLHS2 = *L. acidophilus 2*, VLHS3 = *L. plantarum 3*, VLHS4 = *L. plantarum 4*). The maximum production rates of lactic acid, H_2O_2, and diacetyl by the *Lactobacillus* strains from diseased patients were 1.46 mg/L, 1.36 mmol/L, and 1.72 mg/L, while those from the healthy subjects were 1.08 mg/L, 1.36 mmol/L, and 0.86 mg/L, respectively (Figures 1–3).

Discussion

VVC, which presents with common symptoms like considerable itching and offensive vaginal discharge (26), and sometimes a burning sensation, is a common infection that affects the quality of life for many women. Generally, the affected women will turn to self-medication with over-the-counter antifungal drugs (e.g., imidazoles, polyenes, and ketoconazoles), which are used as either topical or systemic antifungal agents (6,27). Meanwhile, pathogenic *Candida* species have developed resistance to several antifungal agents (28,29), and another potential limitation of the antifungal drugs is the frequency of their interactions with co-administered drugs, which sometimes results in adverse clinical consequences (30).

The vaginal microflora of healthy asymptomatic women is dominated by diverse species of anaerobic, aerobic, microaerophilic, as well as facultative anaerobic lactobacilli flora (1). Reports characterising and selecting strains of *Lactobacillus* for potential use as probiotics for regenerating the vaginal flora of women with recurrent episodes of bacterial vaginosis indicate that the species recovered were *L. acidophilus*, *L. crispatus*, and *L. delbrueckii* ssp. *delbrueckii* (16,31–33). Similar species (*L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. fermentum, L. jensenii,* and *L. plantarum*) were also recovered from vaginal specimens of both healthy subjects and diseased patients in this study.

The role of *Lactobacillus* species in the female urogenital tract as a barrier to infection is of considerable interest (14) because *Lactobacillus* species are believed to contribute to the control of vaginal microbiota by competing with other microflora for adherence to the vaginal epithelial cells and also by producing antimicrobial compounds such as H_2O_2, organic acids, and bacteriocin-like substances

Table 2: The antimicrobial activity of the cell-free supernatant from the vaginal *Lactobacillus* strains of healthy subjects on the *Candida* strains

***Candida* strain**	**Antimicrobial-producing *Lactobacillus* strain [a]**			
	VLHS1	**VLHS2**	**VLHS**	**VLHS4**
C. albicans				
- AC1	12.0	22.0	15.0	12.0
- AC2	15.0	R	18.0	10.0
- BC1	R	18.0	22.0	12.0
- FC1	10.0	R	R	R
- FC2	10.0	10.0	R	12.0
- GC1	R	10.0	15.0	15.0
- X1C	12.0	10.0	15.0	15.0
- IC	12.0	R	15.0	10.0
- 2C2	10.0	12.0	R	R
- 6C1	10.0	12.0	14.0	12.0
- GC2, 4C2, H2C	R	R	R	R
C. glabrata				
- BC1	14.0	10.0	R	R
- 1C2	12.0	10.0	18.0	12.0
- X7C	R	R	16.0	10.0
- 4C1	16.0	10.0	16.0	19.0
- 1TC	R	R	R	12.0
- HC	12.0	10.0	14.0	12.0
C. pseudotropicalis				
- 2C1	10.0	R	R	22.0
- 6C2	R	10.0	28.0	12.0
- 2C2B	10.0	10.0	R	R
- X7C	R	R	14.0	10.0
- 9C2	10.0	12.0	R	R
C. tropicalis				
- HC	18.0	16.0	R	15.0
- 2TC	10.0	12.0	18.0	12.0
- 6C	R	R	R	18.0
- 9C	10.0	12.0	18.0	12.0
- 10C	12.0	14.0	12.0	10.0
- ITC2	12.0	10.0	R	14.0
- 2TC2	12.0	10.0	12.0	R
- 6C1A	R	R	18.0	10.0
- HC3	12.0	10.0	12.0	12.0
- HC1	14.0	R	R	12.0
- 9CB	10.0	10.0	16.0	18.0
Total % susceptibility	**68.6**	**62.9**	**57.1**	**68.6**

R indicates absence of zone of inhibition (resistance).
[a] VLHS1–2 = *L. acidophilus*, VLHS3–4 = *L. plantarum*.

Table 3: Antagonistic activity of the antimycotic agents on the *Candida* strains

***Candida* strain**	**Antimycotic agent [a]**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
C. albicans									
- IC	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
- 2C2	20.0	R	25.0	25.0	20.0	20.0	20.0	R	20.0
- H2C	30.0	R	R	R	20.0	25.0	22.0	25.0	30.0
- GC1	R	R	30.0	25.0	10.0	35.0	R	10.0	15.0
- FC1	10.0	R	20.0	20.0	R	25.0	R	20.0	20.0
- AC1	10.0	R	30.0	20.0	20.0	30.0	R	20.0	20.0
- AC2	R	10.0	30.0	20.0	10.0	10.0	10.0	10.0	20.0
- BC1	10.0	R	20.0	20.0	10.0	10.0	R	20.0	20.0
- GC2	20.0	10.0	30.0	20.0	20.0	20.0	R	10.0	10.0
- FC2	10.0	10.0	20.0	20.0	20.0	20.0	R	20.0	10.0
- 4C2	R	R	R	R	R	R	R	R	R
- 6C1	10.0	10.0	30.0	10.0	10.0	10.0	10.0	10.0	10.0
C. glabrata									
- 4C1	R	R	25.0	20.0	R	20.0	R	10.0	10.0
- ITC	20.0	20.0	35.0	20.0	20.0	25.0	R	20.0	25.0
- IC2	20.0	10.0	25.0	20.0	10.0	10.0	R	R	R
- HC	10.0	10.0	20.0	20.0	10.0	20.0	10.0	10.0	10.0
- BC1	10.0	20.0	30.0	20.0	10.0	20.0	R	10.0	R
C. pseudotropicalis									
- X7C	10.0	10.0	30.0	20.0	10.0	10.0	R	10.0	10.0
- 6C2	R	R	R	R	R	R	R	R	R
- 2C1	10.0	10.0	30.0	20.0	10.0	15.0	10.0	10.0	10.0
C. tropicalis									
- HC	R	R	30.0	20.0	10.0	20.0	R	10.0	10.0
- *6C*	10.0	10.0	30.0	20.0	10.0	20.0	R	10.0	10.0
- 9C	R	R	25.0	20.0	10.0	10.0	10.0	15.0	10.0
- 10C	10.0	10.0	20.0	20.0	10.0	20.0	R	20.0	10.0
- 2TC	10.0	10.0	20.0	20.0	10.0	10.0	R	10.0	R
- HC1	R	10.0	20.0	10.0	10.0	R	10.0	10.0	10.0
Total % susceptibility	**69.2**	**57.7**	**88.4**	**88.4**	**88.4**	**92.3**	**30.8**	**84.6**	**84.6**

R indicates absence of zone of inhibition (resistance).

[a] 1 = doxycycline capsule, 2 = Mycoten tablet, 3 = Diflucan capsule, 4 = Mycostatin tablet, 5 = Canesten tablet, 6 = Fungoral tablet, 7 = Flagyl tablet, 8 = Canensten cream, 9 = Mycoten cream

(possibly biosurfactants), which lower the vaginal pH (4,16,17,34,35). In this study, we found minimal to moderate inhibition of *Candida* species by antimicrobials (lactic acid, H_2O_2, and diacetyl) produced from *Lactobacillus* species. Lactic acid, one of the inhibitory agents produced by the screened *Lactobacillus* species, is the major end product of the carbohydrate catabolism of lactic acid bacteria. The maximum amount of lactic acid produced by vaginal *Lactobacillus* strains in this study was 27.0 g/L.

H_2O_2, which generates cytotoxic reactive oxygen, superoxide anions, and hydroxyl radicals in the vaginal fluid (36), has been considered

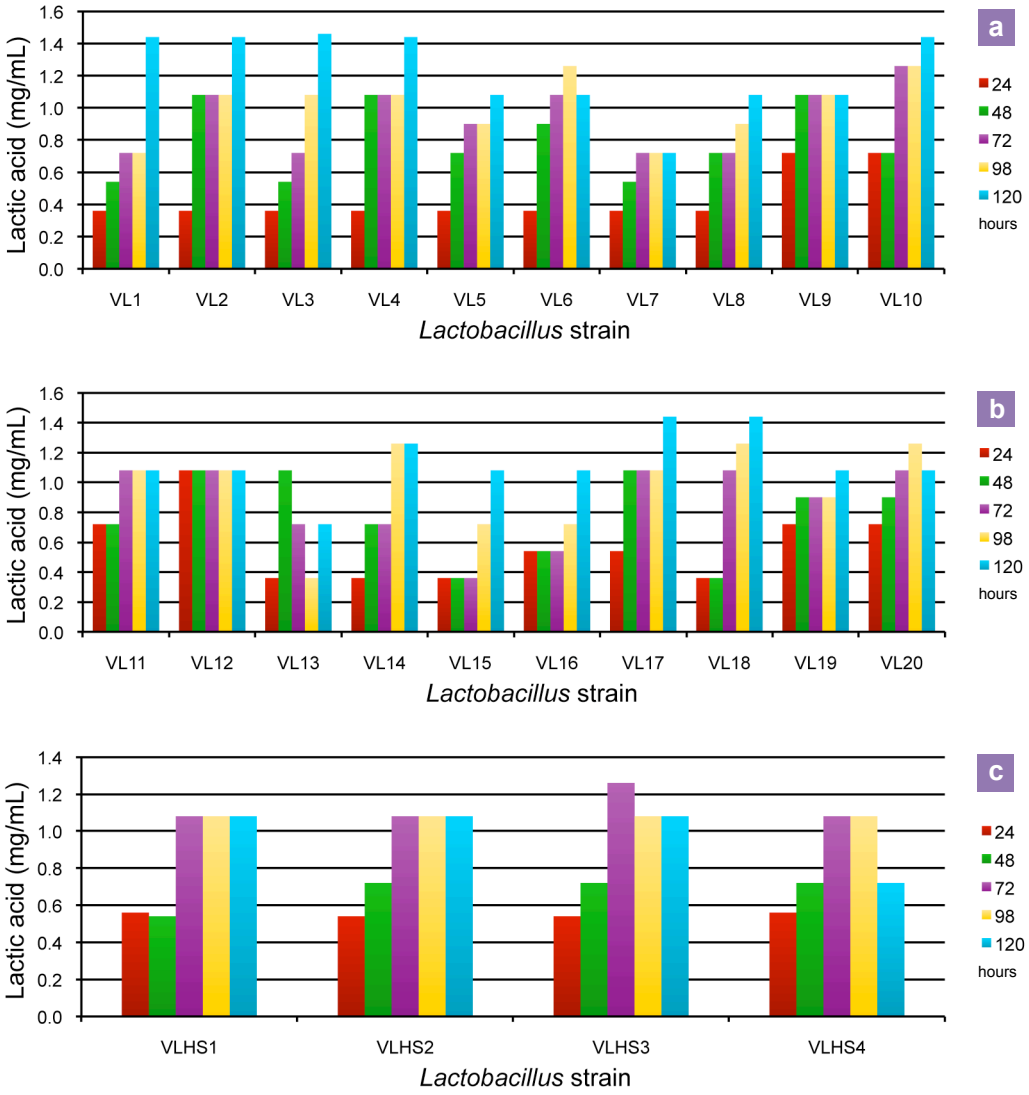


Figure 1: Lactic acid production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

a key factor in the antagonism of pathogens by *Lactobacillus*. Some studies have noted that *Lactobacillus* species do not have a protective role against vaginal candidiasis per se (16,37,38). However, the metabolic production and release of H_2O_2 by lactic acid bacteria has been reported to be antagonistic towards several other bacteria. Moreover, the accumulated level of H_2O_2 in the culture medium has been found to be auto-inhibitory (39). H_2O_2 inhibits the growth of undesirable microorganisms and may also react with other components to form additional inhibitory compounds. The maximum amount of H_2O_2 produced by the vaginal *Lactobacillus* strains in this study was 1.36 mmol/L, and it was confirmed that the H_2O_2-producing strains were moderately inhibitory against the pathogenic *Candida* species in vitro. Moderate inhibition due to H_2O_2 production has been reported previously (33,40).

In this study, all of the *Lactobacillus* strains produced varying amounts of diacetyl. The maximum amount of diacetyl produced was 0.86 mg/mL, which is consistent with earlier reports, including those of Daeschel (41) and Vandenbergh (42), which found that substances produced by lactic acid bacteria, other than lactic and acetic acids, are produced in much smaller amounts. Diacetyl has also been reported to possess antimicrobial activity (43).

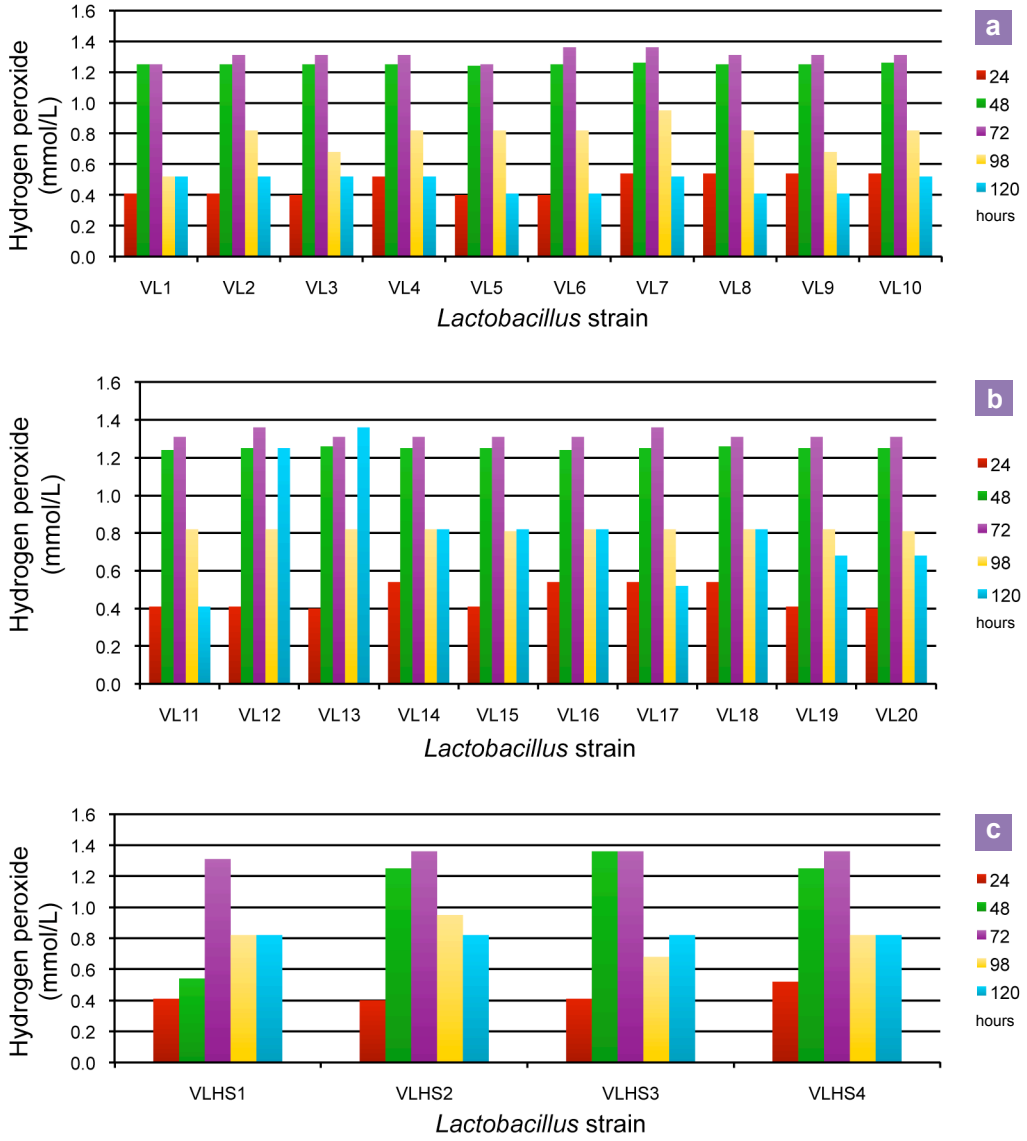


Figure 2: Hydrogen peroxide production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

The ability of citrate-positive *Lactococcus lactis* ssp. *lactis* (formerly *Streptococcus lactis* ssp. *diacetylactis*) to produce diacetyl and acetoin from citrate has led to its widespread use as a characteristic aroma-producer in many dairy products (44). Although not conclusive, the characteristic aroma produced by lactic acid bacteria, due to diacetyl and acetoin, may be responsible for the better smell of the *Lactobacillus* strains obtained from the healthy subjects as compared with the pungent smell from the diseased patients.

It has been reported that hormonal changes predispose women to vaginal candidiasis (45). It was also confirmed that oral contraceptives may influence the recurrence of symptomatic VVC (46), and according to Maccato et al. (6), women who use high doses of oral contraceptives, contraceptive sponges, and antibiotics are at increased risk of colonisation and symptomatic vaginitis. The results obtained in the study of Fidel et al. (47) suggested that oestrogen, but not progesterone, is an important factor in hormone-associated susceptibility to *C. albicans* vaginitis; the use of oral or injectable hormonal contraception has been found to alter susceptibility to sexually transmitted diseases. Meanwhile, a number of females, especially non-literate women and those

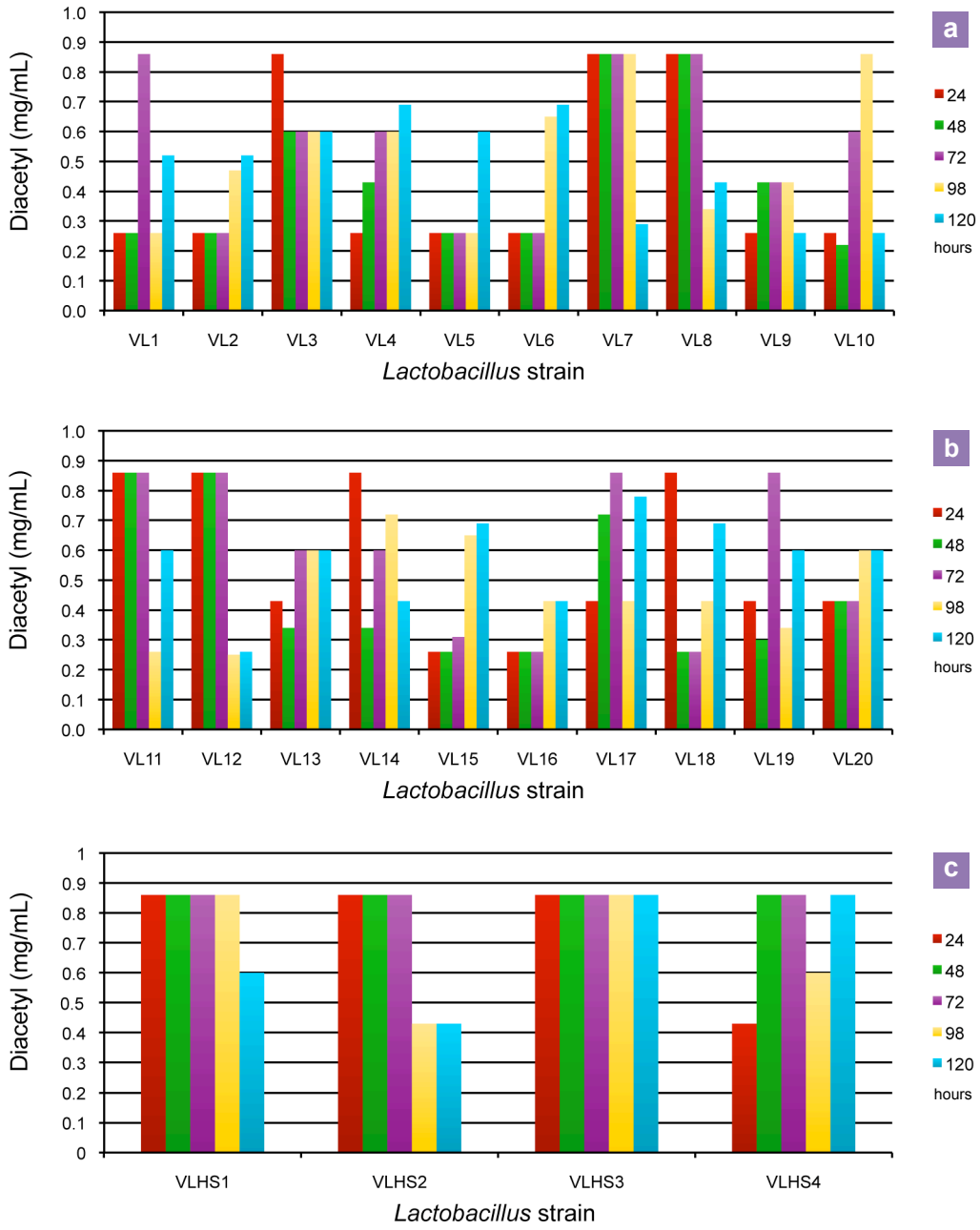


Figure 3: Diacetyl production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

who work in the sex trade in Nigeria, believe that contraceptives prevent sexually transmitted infections in addition to preventing pregnancy.

Although the role of reproductive hormones in the acquisition of vaginal candidiasis remains unclear (47), a study by Baeten et al. (48) found that users of oral contraceptives were at an increased risk for *Chlamydia* infection and vaginal candidiasis when compared with women who were not using contraception. Oestrogen was found to reduce the ability of vaginal epithelial cells to inhibit the growth of *C. albicans* (47), which is contradictory to the assertion by some women, such as the sexual workers mentioned above, that contraceptives prevent sexually transmitted infections. As reported in this study, none of the most commonly available contraceptives in the country inhibited the *Candida* strains in vitro.

In a review article, Apisarnthanarax et al. (49) even noted that the use of combined oestrogenic oral contraceptives was more commonly associated with candidiasis. The results obtained in the present study therefore confirm that commonly available oral contraceptives in Nigeria have no in vitro inhibitory effect on pathogenic *Candida* strains isolated from clinical cases of candidiasis.

Conclusion

Basic knowledge of the vaginal ecosystem and new research can lead to a successful therapeutic approach (4). The empirical use of inhibitory microorganisms, as highlighted by the findings of this study, indicate that lactobacilli can be considered a potential adjunct bio-therapeutic agent in women with VVC, especially for those with resistant strains or those who have adverse effects or contraindications when using antifungal agents. The inhibitory activities of the vaginal *Lactobacillus* species must have been synergistically enhanced by the production of antimicrobials. The protective role of lactobacilli in preventing VVC is controversial because the recovery of antimicrobial-producing lactobacilli can be dependent on diet and geographical location. Therefore, it is very important that further studies be performed on additional indigenous *Lactobacillus* strains to determine their clinical potential for the adjunct treatment of VVC.

Authors' Contributions

Conception and design, critical revision of the article: AAOO
Provision of study materials: AAOO, MAO
Collection and assembly of data: AAOO, VBB
Analysis and interpretation of the data, drafting and final approval of the article: AAOO, MAO, VBB

Correspondence

Dr Adenike Adedayo O Ogunshe
BSc, MSc, PhD (Ibadan)
Applied Microbiology and Infectious Diseases,
Department of Microbiology, Faculty of Science,
University of Ibadan,
Ibadan, Oyo State, 900001
Nigeria
Fax: +234-2-8103043
Email: adenikemicro@yahoo.com

References

1. Redondo-Lopez V, Cook RL, Sobel JD. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Rev Infect Dis*. 1990;**12(5)**:856–872.
2. Boris S, Suarez JE, Vazquez F, Barbes. C. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect Immun*. 1998;**66(5)**:1985–1989.
3. Onderdonk AB, Delaney ML, Hinkson PL. DuBois AM. Quantitative and qualitative effects of douche preparations on vaginal microflora. *Obstet Gynecol*. 1992;**80(3 Pt 1)**:333–338.
4. Kunz J. Causes and consequences of an unbalanced vaginal ecosystem. *Praxis* (*Bern 1994*). 1995;**84(48)**:1405–1415.
5. Ogunshe AA, Bakare RA, Fasina N. Microbial pathogens implicated in reproductive health infections in a special treatment clinic in Ibadan, Nigeria. *J Fam Repr Health*. 2009;**3(1)**:9–17.
6. Maccato ML, Kaufman RH. Fungal vulvovaginitis. *Curr Opin Obstet Gynecol*. 1991;**3(6)**:849–852.
7. Geiger AM, Foxman B, Sobel JD. Chronic vulvovaginal candidiasis: Characteristics of women with Candida albicans, C. glabrata and no Candida. *Genitourin Med*. 1995;**71(5)**:304–307.
8. Ono F, Yasumoto S. Genital candidiasis. *Nippon Rinsho*. 2009;**67(1)**:157–161.
9. Carlson P, Richardson M, Paavonen J. Evaluation of the Oricult-N dipslide for laboratory diagnosis of vaginal candidiasis. *J Clin Microbio*l. 2000;**38(3)**: 1063–1065.
10. Klepser ME. Antifungal resistance among *Candida* species. *Pharmacotherapy*. 2001; **21(8 Pt 2)**:124S–132S.
11. Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: Molecular mechanisms and clinical consequences. *Lancet Infect Dis*. 2002;**2(2)**:73–85.
12. Mishra NN, Prasad T, Sharma N, Payasi A, Prasad R, Gupta DK, et al. Pathogenicity and drug resistance in *Candida albicans* and other yeast species. A review. *Acta Microbiol Immunol Hung*. 2007;**54(3)**: 201–235.
13. Ljungh A, Wadstrom T, editor. *Lactobacillus molecular biology*: *From genomics to probiotics*. Norfolk (UK): Caister Academic Press; 2009.
14. Boris S, Barbes C. Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes Infect*. 2000;**2(5)**:543–546.
15. Jin L, Tao L, Pavlova SI, So JS, Kiwanuka N, Namukwaya Z, et al. Species diversity and relative abundance of vaginal lactic acid bacteria from women in Uganda and Korea. *J Appl Microbiol*. 2007;**102(4)**:1107–1115.

16. Martinez RC, Franceschini SA, Patta MC, Quintana SM, Nunes AC, Moreira JL, et al. Analysis of vaginal lactobacilli from healthy and infected Brazilian women. *Appl Environ Microbiol*. 2008;**74(14)**: 4539–4542.

17. McLean NW, Rosenstein IJ. Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. *J Med Microbiol*. 2000;**49(6)**:543–552.

18. Osset J, Garcia E, Bartolome RM, Andreu A. Role of *Lactobacillus* as protector against vaginal candidiasis. *Med Clin (Barc)*. 2001;**117(8)**:285–298.

19. Rogosa M, Sharpe ME. Species differentiation of human vaginal lactobacilli. *J Gen Microbiol*. 1960;**23**:197–201.

20. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, et al. In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. *Amer J Clin Nutr*. 2001;**73(2 Suppl)**:386S–392S.

21. Kreger-van Rij NJW, editor. *The yeasts: A taxonomic study*. 3rd ed. Amsterdam (NL): Elsevier Science Publishers; 1984.

22. Ogunshe AAO, Lawal OA, Iheakanwa CI. Effects of simulated preparations of plants used in Nigerian traditional medicine on Candida spp. associated with vaginal candidiasis. *J Ethnobot Res Appl*. 2009;**6**:373–383.

23. Tagg JR, McGiven AR. Assay system for bacteriocins. *Appl Microbiol*. 1971;**21(5)**:943.

24. Horwitz W, editor. *Official methods analysis of the Association of Official Analytical Chemists*. 13th ed. Gaithersburg (MD): Association of Official Analytical Chemists; 1980.

25. Committee on Specification of Food Chemicals Codex of the Committee on Food Protection, National Research Council. *Food chemicals codex*. 2nd ed. Washington (DC): National Academy of Sciences; 1972.

26. Sobel JD. Vulvovaginal candidosis. *Lancet*. 2007;**368(9577)**:1961–1971.

27. Mardh PA, Wagstrom J, Landgren M, Holmen J. Usage of antifungal drugs for therapy of genital *Candida* infections, purchased as over-the-counter products or by prescription: I. Analyses of a unique database. *Infect Dis Obstet Gynec*ol. 2004;**12(2)**: 91–97.

28. Odds FC. Antifungal agents: Resistance and rational use. In: Gould IM, van der Meer JWM, editors. *Antibiotic policies: Theory and practice*. Gould IM, van der Meer JWM, editors. New York (NY): Kluwer Academic/Plenum Publishers; 2005. p. 311–330.

29. Panizo MM, Reviakina V, Dolande M, Selgrad S. *Candida* spp. in vitro susceptibility profile to four antifungal agents. Resistance surveillance study in Venezuelan strains. *Med Mycol*. 2009;**47(2)**: 137–143.

30. Albengres E, Le Louet H, Tillement JP. Systemic antifungal agents. Drug interactions of systemic antifungal agents. *Drug Saf*. 1998;**18(2)**:83–97.

31. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, et al. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J Clin Microbio*l. 1989;**27(2)**:251–256.

32. McLean NW, Rosenstein IJ. Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. *J Med Microbiol*. 2000;**49(6)**:543–552.

33. Martin R, Soberon N. Vaneechoutte M, Florez AB, Vazquez, F Suarez JE. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol*. 2008;**11(4)**:261–266.

34. Kaewsrichan J, Peeyananjarassri K, Kongprasertkit J. Selection and identification of anaerobic lactobacilli producing inhibitory compounds against vaginal pathogens. *FEMS Immunol Med Microbiol*. 2006;**48(1)**:75–83.

35. Mijac VD, Dukic SV, Opavski NZ, Dukic MK, Ranin LT. Hydrogen peroxide producing lactobacilli in women with vaginal infections. *Eur J Obstet Gynecol Reprod Biol*. 2006;**129(1)**:69–76.

36. Klebanoff SJ, Hilier SL, Eschenbach DA, Waltersdorph AM. Control of the microbial flora of the vagina by H_2O_2-generating lactobacilli. *J Infect Dis*. 1991;**164(1)**:94–100.

37. Pascual LM, Daniele MB, Pajaro C, Barberis L. *Lactobacillus* species isolated from the vagina: Identification, hydrogen peroxide production and nonoxynol-9 resistance. *Contraception*. 2006;**73(1)**:78–81.

38. Chassot F, Camacho DP, Patussi, EV, Donatti L, Svidzinski TI, Consolaro ME. Can *Lactobacillus acidophilus* influence the adhesion capacity of *Candida albicans* on the combined contraceptive vaginal ring? Contraception. 2010;**81(4)**:331–335.

39. Wheater DM, Hirsch A, Mattick AT. Possible identity of lactobacillin with hydrogen peroxide produced by lactobacilli. *Nature*. 1952;**170(4328)**:623–624.

40. Hillier SL, Krohn MA, Klebanoff SJ, Eschenbach DA. The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. *Obstet Gynecol*. 1992;**79(3)**:369–373.

41. Daeschel AM. Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol*. 1989;**43**:91–94.

42. Vandenbergh PA. Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol Revs*. 1993;**12(1–3)**:221–237.

43. Lanciotti R, Patrignani F, Bagnolini F, Guerzoni ME, Gardini F. Evaluation of diacetyl antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. *Food Microbiol*. 2003;**20(5)**:537–543.

44. Kaneko T, Waranabe Y, Suzuki H. Enhancement of diacetyl production by a diacetyl-resistant mutant of citrate-positive *Lactococcus lactis* ssp. *lactis* 3022 and by aerobic conditions of growth. *J Dairy Sci*. 1990;**73(2)**:291–298.

45. Kinsman O, Collard AE. Hormonal factors in vaginal candidiasis in rats. *Infect Immun*. 1986;**53(3)**: 498–504.

46. Spinillo A, Capuzzo E, Nicola S, Baltaro F, Ferrari A, Monaco A. The impact of oral contraception on vulvovaginal candidiasis. *Contraception*. 1995;**51(5)**:293–297.

47. Fidel PL, Cutright J, Steele C. Effects of reproductive hormones on experimental vaginal candidiasis. *Infect Immun*. 2000;**68(2)**:651–657.

48. Baeten JM, Nyange PM, Richardson BA, Lavreys L, Chohan B, Martin HL, et al. Hormonal contraception and risk of sexually transmitted disease acquisition: Results from a prospective study. *Am J Obstet Gynecol*. 2001;**185(2)**:380–385.

49. Apisarnthanarax P, Self SJ, Ramer SG, Montes LF. Oral contraceptives and candidiasis: A review of current data. *Cutis*. 1974;**14**:77–82.

Original Article

Effects of Duration of Diabetes on Behavioural and Cognitive Parameters in Streptozotocin-Induced Juvenile Diabetic Rats

Ravishankar Rajashree[1], Sanjiva D Kholkute[2], Shivaprasad S Goudar[1]

Submitted: 30 May 2011
Accepted: 17 Jul 2011

[1] *Department of Physiology, Jawaharlal Nehru Medical College, Karnataka Lingayat Education University, Belgaum-590010, India*

[2] *National Institute of Research in Reproductive Health, Indian Council of Medical Research, Parel, Mumbai-400012, India*

Abstract

Background: **Diabetic encephalopathy is a recently recognised complication of early-onset type 1 diabetes in children. The abnormalities underlying diabetic encephalopathy are complex and poorly understood, and the impact of disease duration on behavioural and cognitive parameters also remains unclear. Hence, the present study was conducted to determine the effects of different durations of hyperglycaemia on behavioural and cognitive parameters in young streptozotocin-induced diabetic rats.**

Methods: **Diabetes was induced in young, weaned, age-matched rat pups by streptozotocin injection (50 mg/kg body weight, intraperitoneally). Diabetic status was confirmed on post-natal day 30. The rats were tested in the elevated plus maze 10 and 20 days after diabetes induction.**

Results: **Diabetic rats had significantly impaired behavioural and cognitive functions compared with age-matched controls. Increased anxiety levels and cognitive deficits were observed in rats that had been diabetic for 20 days compared with their 10-day counterparts.**

Conclusion: **It is essential to diagnose and treat early-onset type 1 diabetes in young children to prevent irreversible cognitive dysfunction.**

Keywords: *anxiety, cognition, juvenile-onset diabetes mellitus, learning, maze learning, memory, rats*

Introduction

Behavioural and cognitive changes associated with type 1 diabetes mellitus (T1DM) have recently gained attention. Concerns about the deleterious effects of T1DM on the central nervous system have grown with the increasing incidence of T1DM in children (1). Many studies have clearly shown cognitive and behavioural changes in type 1 diabetic rats and humans, which are evident in elevated levels of anxiety, depression, and slowing of mental speed and flexibility (2–5).

Diabetes-induced behavioural and cognitive changes are related to several factors. Both diabetic complications and reduced central serotonin (5-hydroxytryptamine, 5-HT) synthesis and metabolism are thought to underlie behavioural and cognitive dysfunctions in patients with T1DM (6,7). It has become evident that insulin and C-peptide deficiencies, including perturbations of their signalling cascades, lead to cerebral dysmetabolism and interference with the regulation of neurotropic factors and their receptors. Ultimately, this cascade of events leads to neuronal loss, causing profound deficits in behavioural and cognitive functions (8). However, the specific mechanisms underlying these changes and whether they relate to the duration of hyperglycaemia are unknown.

Although the magnitude of most of these cognitive decrements is relatively modest, even moderate behavioural and cognitive changes can potentially hamper the day-to-day activities of a diabetic child. These cognitive decrements may present problems in more demanding situations, and critically, can have a negative impact on the quality of life.

Patients with an onset of diabetes before the age of 5 years may be more sensitive to diabetic complications and diabetic effects related to encephalopathy. Many researchers have shown that there is a relationship between neuropsychological changes and early-onset T1DM (3,9,10). The contributions of several

disease variables, such as diabetic duration, level of glycaemic control and the developmental trajectory of neuropsychological impairments, remain unresolved. Hence, the present study was conducted to evaluate the effects of different diabetic durations on various behavioural and cognitive parameters using the elevated plus maze (EPM) in streptozotocin (STZ)-induced diabetic rat pups.

Materials and Methods

Inbred male and female Wistar rats, 25 days old with weights of 45–50 g, were selected for the study. Experiments were approved by the Institutional Animal Ethical Committee (627/02/a/CPCSEA dated 17 July 2008). The rats were maintained on a 12:12-hour light:dark cycle under controlled temperatures (25 °C, SD 3) and had ad libitum access to food (Amrut feeds, standard rat pellets) and water. All experiments were performed between 08:00 and 16:00 hour. Rats were randomly divided into the following groups of 6 rats each:

- Group 1 (N-10): Control for 10 days
- Group 2 (D-10): Diabetic for 10 days
- Group 3 (N-20): Control for 20 days
- Group 4 (D-20): Diabetic for 20 days

Rats in the diabetic groups received an intraperitoneal injection of STZ (50 mg/kg) on post-natal day 25. Four days later, blood was collected from the tail vein following an overnight fast (11–13). Fasting blood sugar (FBS) was measured with a standard glucometer (Optium, Germany), and the day that diabetes was confirmed was considered to be diabetic day 1. Rats with FBS lower than 200 mg/dL were excluded from the study. Eleven days after diabetes confirmation, rats in the D-10 group were assessed for cognitive and behavioural parameters in the EPM. Similarly, rats in the D-20 group were also assessed with the same measures 21 days after diabetes confirmation. The details of the EPM test are explained below.

The EPM is widely used for rodent neuropsychological assays, such as anxiety behaviour as well as learning and memory tests, and valid results can be obtained in a short, 5-minute testing period. The maze consists of 4 arms (2 open arms without walls, OAs, and 2 arms enclosed by 30-cm-high walls, EAs), 50 cm long and 10 cm wide, that are attached to a central platform (5 × 5 cm) at right angles. The apparatus is elevated to a height of 50 cm above the floor and is kept in a brightly lit room.

Anxiety protocol

The rats were placed on the central platform with their heads oriented towards an OA. The frequency of entries into the OAs and EAs were scored and time spent in the OAs was recorded for 5 minutes. The number of entries into the OAs of the maze and the time spent in those arms are the measures of anxiety, and decreases in these measures indicate an anxiogenic effect. The number of EA entries serves as the measure of locomotor activity in this test. An arm entry is defined as all 4 paws entering an arm, and an arm exit is defined as 2 paws leaving an arm. During this period, ethological parameters such as number of rears, grooming, and boli of excreta were also counted.

Learning protocol

A line was drawn to divide the EA into 2 equal parts. On days 1 and 2, we measured the time it took for each rat to cross the line in the EA (transfer latency). The rat was initially placed at the end of an OA and allowed to explore for 90 seconds. The rat was required to have its body and 4 paws cross the line in the EA; if the rat did not cross the line after the time limit, it was manually placed beyond the line and transfer latency was recorded as 90 seconds. After crossing the line, the rat was allowed to spend 30 seconds exploring the apparatus. Learning was defined as reduced transfer latency on day 2 compared with day 1. Over the test period, normal rats typically cross the line in the EA more quickly on day 2 than on day 1 (14–17).

Statistical analysis

The results are expressed as means and standard deviations (SD). The between-group comparisons of FBS were made with unpaired Student's *t* tests. For behavioural measures from the EPM, the between-group comparisons were made with Mann–Whitney U non-parametric tests. Differences were considered significant at $P < 0.05$.

Results

We randomly assigned 72 rats to different experimental groups. There were 6 rats in each of the control groups (N-10 and N-20). The remaining 50 rats were given STZ injections to induce diabetes. Out of the 50 rats, 22 died and 28 became diabetic; 15 of the diabetic rats achieved the required diabetic state (FBS greater than 200 mg/dL) and were included in the study (D-10 and D-20). During the study period, 3 of

the diabetic rats died. Data collected from the 4 groups of rats, control (N-10 and N-20) and diabetic (D-10 and D-20), are summarised below.

FBS was measured on post-natal days 30 and 60, and the results are shown in Table 1. A statistically significant difference ($P < 0.001$) observed in FBS values on post-natal day 30 between diabetic rats and their respective age-matched controls. The severity of diabetes increased over time, showing significant differences in FBS levels between post-natal days 30 and 60 ($P = 0.018$) in diabetic rats with a 20-day hyperglycaemia duration. The diabetic rats in the D-10 group were sacrificed immediately after the tests and processed further for neurohistological studies.

The EPM performances are shown in Table 2. Anxiety tests indicate that the diabetic rats spent less time in the OA and made fewer arm entries compared with the control rats. The statistically significant differences were observed in the number of OA entries ($P = 0.009$) and the time spent in the OA ($P = 0.006$) between D-20 rats and their age-matched controls. D-10 rats showed no showed no significant differences compared with their age-matched controls. No significant differences were observed in other behavioural (ethological) parameters, such as rearing, grooming, and number of boli excreted in both in both D-10 and D-20 groups compared with their age-matched controls. Furthermore, the number of EA entries did not differ between

Table 1: The effects of diabetes duration on fasting blood sugar (FBS) level in normal control and streptozotocin-induced diabetic rats

Group	FBS (mg/dL)	
	Post-natal day 30	Post-natal day 60
Control—10 days	87.3 (3.85)	-
Diabetic—10 days	233.0 (10.27) [a]	-
Control—20 days	86.8 (3.37)	89.0 (3.95)
Diabetic—20 days	269.1 (20.41) [a]	319.3 (38.51) [a,b]

Each group consisted of 6 rats. All values are expressed as mean (SD).
[a] Significant difference ($P < 0.05$) compared with the respective normal control by unpaired Student's *t* tests.
[b] Significant difference ($P < 0.05$) compared with the post-natal day 30 by paired Student's *t* tests.

Table 2: The effects of diabetes duration on anxiety level in control and streptozotocin-induced diabetic rats in the elevated plus maze

Group	No. of entries		Time in OA (seconds)	Ethological parameters		
	EA	OA		Rearing	Grooming	No. of boli excreted
Control—10 days	4.1 (0.98)	1.83 (0.16)	13 (0.77)	6.1 (0.47)	2.6 (0.49)	1.1 (0.30)
Diabetic—10 days	3.3 (0.33)	0.83 (0.75)	6.16 (3.09)	4.8 (0.60)	3.1 (0.47)	1.8 (0.60)
Control—20 days	6.6 (0.61)	4.3 (1.23)	64.3 (20.87)	9.6 (0.88)	2.1 (0.47)	0.1 (0.16)
Diabetic —20 days	5.6 (0.92) [b]	1 (0.36) [a,b]	9.5 (3.66) [a]	8.1 (0.94) [b]	3.3 (0.33)	0.8 (0.47) [b]

Each group consisted of 6 rats. All values are expressed as mean (SD).
[a] Significant difference ($P < 0.05$) compared with the respective normal control by Mann–Whitney non-parametric tests.
[b] Significant difference ($P < 0.05$) compared with the 10-day diabetic rats by Mann–Whitney non-parametric tests.
Abbreviations: EA = enclosed arm, OA = open arm.

diabetic and normal rats. Rats in the D-20 group differed significantly from D-10 group in the number of entries into EA (P = 0.002) and into OA (P = 0.004), as well the numbers of rears (P < 0.001) and boli of excreta (P = 0.009).

The transfer latency results are summarised in Table 3. On day 1, there were significant differences in the transfer latency results of D-10 (P = 0.039) and D-20 (P = 0.006) rats compared with their respective controls on day 1 in the learning paradigm. Significant differences were also observed on day 2 in D-10 (P = 0.009) and D-20 (P = 0.02) rats compared with the controls in the memory retention trials. In addition, the transfer latencies were significantly difference between D-10 and D-20 groups on both day 1 (P = 0.03) and day 2 (P = 0.013).

Discussion

STZ-induced diabetic rat is a well-established animal model of diabetes. Intra-peritoneal injection of STZ induces "chemical diabetes" in a wide variety of animal species, including rats, by selectively damaging the insulin-secreting β cells of the pancreas, as evidenced by their clinical symptoms of hyperglycaemia and hypoinsulinaemia (18).

The EPM is a widely accepted test in the study of anxiety in rodents and other animal models (17,19–24). The EPM is also sensitive enough to detect deficits in associative learning and memory in rats (14).

The EPM results revealed increased anxiety in diabetic rats compared with control rats, which was evident in the decreased number of OA entries and less time spent in the OA. However, no significant differences in the number of EA entries were observed, suggesting no gross locomotor activity changes in the diabetic rats. The ethological measures such as rears, grooming, and number of excreted boli also did not differ significantly between groups, although modest differences were observed. Significant differences were seen between rats in D-10 and D-20 groups. The increased anxiety levels associated with longer diabetic duration might have worsened cerebral dysmetabolism. Many studies have stated that anxiety in diabetic rats could be attributed to 5-HT, adenylyl cyclase type VIII, and tuberoinfundibular peptide of 39 residues deficiencies (7,8,25,26).

The EPM learning and memory measures from day-1 and day-2 trials showed that the groups of diabetic rats differed significantly, not only compared with normal controls, but also between themselves (D-10 and D-20). This clearly suggests that cognitive decline worsens with increasing duration of hyperglycaemia. The condition of rats in the D-20 group is approximately equivalent to 2 years of diabetes in a human life, and they showed increased cognitive deficits compared with their 10-day counterparts. Many studies link these diabetic cognitive deficits to hyperglycaemia-induced end-organ neuronal damage, dyslipidaemia, amyloidopathy, and tauopathy, among other causes (27–29). In the present study, diabetic rats did not receive any intervention, such as insulin, that would have prevented the neuronal damage. Hence, untreated hyperglycaemia for long durations may be one cause of diabetic encephalopathy. Other consequences of insulin deficits and perturbations include innate inflammatory responses affecting synaptogenesis and neuronal degeneration. Eventually, this cascade of events leads to more profound deficits in behavioural and cognitive functions due to extensive neuronal loss and decreased white matter density of myelinated cells. Neuroimaging data suggest white matter

Table 3: The effects of diabetes duration on learning and memory in control and STZ-induced diabetic rats in the elevated plus maze

Group	Transfer latency (seconds)	
	Day 1	Day 2
Control—10 days	63.0 (6.32)	25.0 (4.83)
Diabetic—10 days	81.0 (4.18) [a]	60.0 (9.69) [a]
Control—20 days	46.6 (10.07)	14.1 (2.28)
Diabetic—20 days	87.8 (1.51) [a, b]	72.8 (14.00) [a, b]

Each group consisted of 6 rats. All values are expressed as mean (SD).
[a] Significant difference (P < 0.05) compared with the respective normal control by Mann-Whitney non-parametric tests.
[b] Significant differences (P < 0.05) compared with the 10-day diabetic rats by Mann-Whitney non-parametric tests.

atrophy in the frontal and temporal brain regions, which could be linked to deficits in certain cognitive domains such as memory, information processing speed, executive function, attention, and motor skill speed. Morphological studies of children with diabetic onset before the age of 6 have revealed a high incidence of mesial temporal lobe sclerosis, which is not associated with a history of hypoglycaemia (8,30). Interestingly, deficits in such cognitive functions are also associated with impaired functional connectivity, which is a measure of functional interactions among brain regions (31). Field excitatory post-synaptic potentials recorded from hippocampal slices of diabetic rats show defects in the induction of hippocampal synaptic plasticity that are linked to difficulties in learning and memory (32).

The present study was designed to investigate the effects of different diabetic durations on behavioural and cognitive dysfunction in early life stages. Using an STZ-induced diabetic model, we found that, in young rats, the diabetic duration significantly contributes to learning and memory deficits, which were irreversible, and to the induction of high levels of anxiety.

Conclusion

It has recently become clear that the central nervous system is not spared from the deleterious effects of diabetes. Diabetic encephalopathy is primarily caused by the direct metabolic perturbations of hyperglycaemia, insulin deficiency, or hypoinsulinaemia. Secondary diabetic encephalopathy occurs as a result of micro- and macrovascular disorders or due to repeated episodes of hypoglycaemia induced by excess insulin (33–35). The results of this study suggest that behavioural and cognitive changes are directly related to the duration of the diabetic state; however, the underlying mechanisms remain unknown. This study highlights the clinical importance of early diagnosis and treatment of juvenile diabetes and associated neuropsychological deficits in children.

Acknowledgements

We are grateful to our fellow statistician Shri S D Mallapur for helping in the data analysis, and Professor Kamarudin Jaalam, Deputy Dean, Universiti Sains Malaysia–Karnataka Lingayat Education International Programme, Belgaum, India, for his timely guidance and encouragement in publishing our research findings.

Authors' Contributions

Conception and design, critical revision and final approval of the article: RR, SDK, SSG
Obtaining of funding, provision of study materials, collection, assembly, analysis, and interpretation of the data, statistical expertise, drafting of the article: RR

Correspondence

Dr Rajashree Ravishankar
BAMS (Karnatak University), MSc, PhD
Jawaharlal Nehru Medical College
Karnataka Lingayat Education University
Belgaum-590010
India
Tel: +91 0831-2455557, 2495555
Fax: +91 0831-2455558
Email: rajashreeravishankar@gmail.com,
shreeravi20@yahoo.com

References

1. Adeghate E, Schattner P, Dunn E. An update on the etiology and epidemiology of diabetes mellitus. *Ann N Y Acad Sci*. 2006; **1084**:1–29.
2. Ramanathan M, Jaiswal AK, Bhattacharya SK. Differential effects of diazepam on anxiety in streptozotocin induced diabetic and non-diabetic rats. *Psychopharmacology (Berl)*. 1998;**135(4)**:361–367.
3. Kuhad A, Chopra K. Curcumin attenuate diabetic encephalopathy in rats: Behavioral and biochemical evidences. *Eur J Pharmacol*. 2007;**576(1–3)**:34–42.
4. Alvarez EO, Beauquis J, Revsin Y, Banzan AM, Roig P, De Nicola AF, et al. Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes. *Behav Brain Res*. 2009;**198(1)**:224–230.
5. Brands AM, Biessels GJ, De Haan EH, Kappelle LJ, Kessels RP. The effects of type 1 diabetes on cognitive performance: A meta-analysis. *Diabetes Care*. 2005;**28(3)**:726 –735.
6. Ryan CM, Williams TM. Effects of insulin-dependent diabetes on learning and memory efficiency in adults. *J Clin Exp Neuropsychol*. 1993;**15(5)**:685–700.
7. Thorre K, Chaouloff F, Sarre S, Meeusen R, Ebinger G, Michotte Y. Differential effects of restraint stress on hippocampal 5-HT metabolism and extracellular levels of 5-HT in streptozotocin-diabetic rats. *Brain Res*. 1997;**772(1–2)**:209–216.
8. Sima AA, Zhang W, Muzik O, Kreipke CW, Rafols JA, Hoffman WH. Sequential abnormalities in type 1 diabetic encephalopathy and the effects of C-Peptide. *Rev Diabet Stud*. 2009;**6(3)**:211–222.
9. Toth C, Schmidt AM, Tuor UI, Francis G, Foniok T, Brussee V, et al. Diabetes, leukoencephalopathy and rage. *Neurobiol Dis*. 2006;**23(2)**:445–461.

10. Schoenle EJ, Schoenle D, Molinari L, Largo RH. Impaired intellectual development in children with Type I diabetes: Association with HbA(1c), age at diagnosis and sex. *Diabetologia*. 2002;**45(1)**: 108–114.

11. Kulkarni SK. *Hand book of experimental pharmacology*. 3rd ed. Delhi (IN): Vallabh Prakashan; 1999.

12. Meiri N, Chelardini C, Tesco G, Galeotti N, Dahl D, Tomsic D, et al. Reversible antisense inhibition of Shaker-like Kv1.1 potassium channel expression impairs associative memory in mouse and rat. *Proc Natl Acad Sci U S A*. 1997;**94(9)**:4430–4434.

13. Walther T, Balschun D, Voigt JP, Fink H, Zuschratter W, Birchmeier C, et al. Sustained long term potentiation and anxiety in mice lacking the *Mas* protooncogene. *J Biol Chem*. 1998;**273(19)**: 11867–11873.

14. Carrie I, Clement M, de Javel D, Frances H, Bourre JM. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J Lipid Res*. 2000;**41(3)**:473–480.

15. Bures J, Buresova O, Huston JP. *Techniques and basic experiments for the study of brain and behavior*. 2nd ed. Amsterdam (NL): Elsevier Science; 1983.

16. Nayak V, Patil PA. Antidepressant activity of fosinopril, ramipril and losartan but not of lisinopril in depressive paradigms of albino rats and mice. *Indian J Exp Biol*. 2008;**46(3)**:180–184.

17. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods*. 1985;**14(3)**:149–167.

18. Hofteizer B, Carpenter AM. Comparison of streptozotocin and alloxan-induced diabetes in rats, including volumetric quantization of pancreatic islet cells. *Diabetologia*. 1973;**9(3)**:178–184.

19. Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav R*. 1997;**21(6)**: 801–810.

20. Cavalli J, Bertoglio LJ, Carobrez AP. Pentylenetetrazole as an unconditioned stimulus for olfactory and contextual fear conditioning in rats. *Neurobiol Learn Mem*. 2009;**92(4)**:512–518.

21. Hansen SL, Sperling BB, Sanchez C. Anticonvulsant and antiepileptogenic effects of GABA receptor ligands in pentylenetetrazole-kindled mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2004;**28(1)**:105–113.

22. De Carvalho RS, Duarte FS, de Lima TC. Involvement of GABAergic non-benzodiazepine sites in the anxiolytic-like and sedative effects of the flavonoid baicalein in mice. *Behav Brain Res*. 2011;**221(1)**: 75–82.

23. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)*. 1987;**92(2)**:180–185.

24. Setem J, Pinheiro AP, Motta VA, Morato S, Cruz AP. Ethopharmacological analysis of 5-HT ligands on the rat elevated plus-maze. *Pharmacol Biochem Behav*. 1999;**62(3)**:515–521.

25. Schaefer ML, Wong ST, Wozniak DF, Muglia LM, Liauw JA, Zhuo M, et al. Altered stress-induced anxiety in adenylyl cyclase type VIII-deficient mice. *J Neurosci*. 2000;**20(13)**:4809–4820.

26. Fegley DB, Holmes A, Riordan T, Faber CA, Weiss JR, Ma S, et al. Increased fear- and stress-related anxiety-like behavior in mice lacking tuberoinfundibular peptide of 39 residues. *Genes Brain Behav*. 2008;**7(8)**:933–942.

27. Holmes CS, Hayford JT, Gonzalez JL, Weydert JA. A survey of cognitive functioning at difference glucose levels in diabetic persons. *Diabetes Care*. 1983;**6(2)**:180– 185.

28. Kim B, Backus C, Oh S, Hayes JM, Feldman EL. Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. *Endocrinology*. 2009;**150(12)**:5294–5301.

29. Heikkila O, Lundbom N, Timonen M, Groop PH, Heikkinen S, Makimattila S. Hyperglycaemia is associated with changes in the regional concentrations of glucose and myo-inositol within the brain. *Diabetologia*. 2009;**52(3)**:534–540.

30. Ho MS, Weller NJ, Ives FJ, Carne CL, Murray K, Vanden Driesen RI, et.al. Prevalence of structural central nervous system abnormalities in early-onset type 1 diabetes mellitus. *J Pediatr*. 2008;**153(3)**:385–390.

31. Van Duinkerkeu E, Klein M, Schoonenboom NS, Hoogma RP, Moll AC, Snoek FJ, et al. Functional brain connectivity and neurocognitive functioning in patients with longstanding type 1 diabetes with and without microvascular complications: A magnetoencephalography study. *Diabetes*. 2009;**58(10)**:2335–2343.

32. Kamal A, Biessels GJ, Gispen WH, Ramakers GM. Synaptic transmission changes in the pyramidal cells of the hippocampus in streptozotocin-induced diabetes mellitus in rats. *Brain Res*. 2006;**1073–1074**:276–80.

33. Sima AA, Kamiya H, Li ZG. Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes. *Eur J Pharmacol*. 2004;**490(1–3)**:187–197.

34. Biessels GJ, Deary TJ, Ryan CM. Cognition and diabetes: A lifespan perspective. *Lancet Neurol*. 2008;**7(2)**:184–190.

35. Biessels GJ. Diabetic encephalopathy. In: Veves A, Malik RA, editors. *Diabetic neuropathy: Clinical management*. 2nd ed. Totowa (NJ): Humana Press Inc.; 2007. p. 187–205.

Original Article

Using Pharmacoeconomic Modelling to Determine Value-Based Pricing for New Pharmaceuticals in Malaysia

George Dranitsaris[1], Ilse Truter[1], Martie S Lubbe[2], Nitin N Sriramanakoppa[3], Vivian M Mendonca[3], Sangameshwar B Mahagaonkar[3]

Submitted: 25 Nov 2010
Accepted: 20 Jun 2011

[1] *Department of Pharmacy, Nelson Mandela Metropolitan University, PO Box 77000, Port Elizabeth, 6031, South Africa*

[2] *School of Pharmacy, North-West University, 53, Borcherds Street, Potchefstroom, 2520, South Africa*

[3] *PharmARC Analytic Solutions, Mercury 2B Block, 6th Floor, Prestige Technology Park, Sarjapur, Marathahalli Outer Ring Road, Bengaluru, Karnataka, 560087, India*

Abstract

Background: **Decision analysis (DA) is commonly used to perform economic evaluations of new pharmaceuticals. Using multiples of Malaysia's per capita 2010 gross domestic product (GDP) as the threshold for economic value as suggested by the World Health Organization (WHO), DA was used to estimate a price per dose for bevacizumab, a drug that provides a 1.4-month survival benefit in patients with metastatic colorectal cancer (mCRC).**

Methods: **A decision model was developed to simulate progression-free and overall survival in mCRC patients receiving chemotherapy with and without bevacizumab. Costs for chemotherapy and management of side effects were obtained from public and private hospitals in Malaysia. Utility estimates, measured as quality-adjusted life years (QALYs), were determined by interviewing 24 oncology nurses using the time trade-off technique. The price per dose was then estimated using a target threshold of US$44 400 per QALY gained, which is 3 times the Malaysian per capita GDP.**

Results: **A cost-effective price for bevacizumab could not be determined because the survival benefit provided was insufficient According to the WHO criteria, if the drug was able to improve survival from 1.4 to 3 or 6 months, the price per dose would be $567 and $1258, respectively.**

Conclusion: **The use of decision modelling for estimating drug pricing is a powerful technique to ensure value for money. Such information is of value to drug manufacturers and formulary committees because it facilitates negotiations for value-based pricing in a given jurisdiction.**

Keywords: *chemotherapy, cost analysis, drug costs, FOLFOX protocol, pharmacoeconomics*

Introduction

The rapid growth of healthcare expenditures has led to increased interest in economic evaluations of healthcare programmes (1). This is particularly true for pharmaceuticals, which constitute a substantial portion of the healthcare budget (2). The basic premise of pharmacoeconomic evaluations (PEs) is to compare the costs and consequences of alternative pharmaceutical interventions and determine which treatment offers the best value for money (3). There are several methods available to evaluate economic efficiency (3,4). All of the approaches measure costs in monetary terms, but differ in how consequences are evaluated.

Decision analysis modelling, one of the most commonly used methods for conducting PEs, is a systematic process that assesses appropriate courses of action in the presence of multiple uncertainties (5). Outcomes are typically presented as the incremental cost per quality-adjusted life year (QALY) gained, which is compared against the value threshold set by national formulary committees. For example, the National Institute of Clinical Excellence in the United Kingdom has established the threshold for drug coverage at £30 000 per QALY gained (6). In the Netherlands, the unofficial threshold is €18 000 per QALY (7). However, these thresholds for economic value do not consider the wealth of the nation.

To address this, the World Health Organization (WHO) has proposed using multiples of a country's per capita gross domestic product, GDP (8,9). Based on the WHO criteria, products more than 3 times the GDP are considered cost ineffective (8,9). Using Malaysia as an example (i.e., per capita GDP for 2010 of US$14 800), the threshold for cost-effectiveness of new drugs would be $44 400 per QALY (10).

Most PEs are conducted with an established product price to estimate the cost per QALY gained. PEs can also be very informative for determining a drug price based on recommended thresholds for economic value. To illustrate the application of PE, we used decision analysis modelling to estimate a price for a cancer drug in Malaysia using the WHO criteria for cost-effectiveness. The drug selected for the case study was bevacizumab, an agent that provides a 1.4-month survival gain when added to first-line chemotherapy in patients with metastatic colorectal cancer, mCRC (11). Bevacizumab was chosen because it has a high cost of acquisition and its economic value has been questioned in recent PE studies (12,13).

Materials and Methods

Economic model

The mCRC was chosen for this analysis because the sequential use of specific chemotherapy regimens for treatment is well established. In patients with mCRC, randomised trials have demonstrated that irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) in combination with infusional 5-fluorouracil (5-FU) and leucovorin is highly active and superior to the previous standard of 5-FU/leucovorin alone (14,15). Data from a large randomised trial verified that sequential schedules of FOLFOX and FOLFIRI (or in the reverse order) are equally effective and have emerged as the first- and second-line standards of care for patients with mCRC (16). Clinical practice guidelines also recommend the addition of an anti-vascular endothelial growth factor (VEGF) such as bevacizumab at some point during chemotherapy for mCRC (17). FOLFOX, FOLFIRI, and bevacizumab are all available in Malaysia.

The two most common methods used to model the clinical and economic consequences of cancer therapy are decision trees and Markov modelling. The former method is used in situations where uncertainly arises once over a period of time. However, in cases where events occur repeatedly, Markov processes are better able to capture the uncertainties that are faced iteratively (18). However, one of the disadvantages of Markov modelling is is that it requires an extensive amount of detailed data. To construct a Markov model of multiple cycles of FOLFOX and FOLFIRI, disease progression and toxicity data would be required for each cycle of chemotherapy. Unfortunately, such data are not available from published clinical trials. Since only aggregate data were available (i.e., median number of cycles of chemotherapy), a decision tree approach was used for the current study.

A decision model for the sequential treatment of mCRC with FOLFOX (± an anti-VEGF) followed by FOLFIRI upon disease progression (Figure 1) was developed with DATA software (Treeage Software Inc., Williamstown, MA). The analytic timeframe was from the first cycle of FOLFOX chemotherapy until death. Perspectives from both the public and private Malaysian health care systems were evaluated. Based on the Response Evaluation Criteria in Solid Tumours, the primary outcome for measuring successful initial therapy was clinical benefit, which was defined as complete tumour response (CR), partial response (PR), or stable disease (SD). Three clinical oncologists, each with experience in treating colorectal cancer, evaluated the face and content validity of the model.

The model (Figure 1) began at the decision node (square) where the first-line treatment choice was either FOLFOX + the "new drug" (bevacizumab) or FOLFOX alone (Figure 1). During the first 2 cycles of chemotherapy, patients were assessed for intolerable toxicity. For patients with severe toxicity, first-line therapy was discontinued in its entirety, and second-line FOLFIRI was offered until disease progression. Upon progression, all patients received best supportive care until death. In contrast, patients not experiencing severe toxicity from first-line FOLFOX (± bavacizumab) continued treatment until disease progression. They were offered second-line FOLFIRI alone, and bevacizumab was discontinued. Upon progression, all patients received best supportive care until death. Epidermal growth factor receptor (EGFR) inhibitors such as cetuximab in mCRC patients with KRAS wild-type tumours were not considered because we did not want to overcomplicate the modelling. Furthermore, these agents would be available to both treatment options in the model, so their inclusion would not impact the final results.

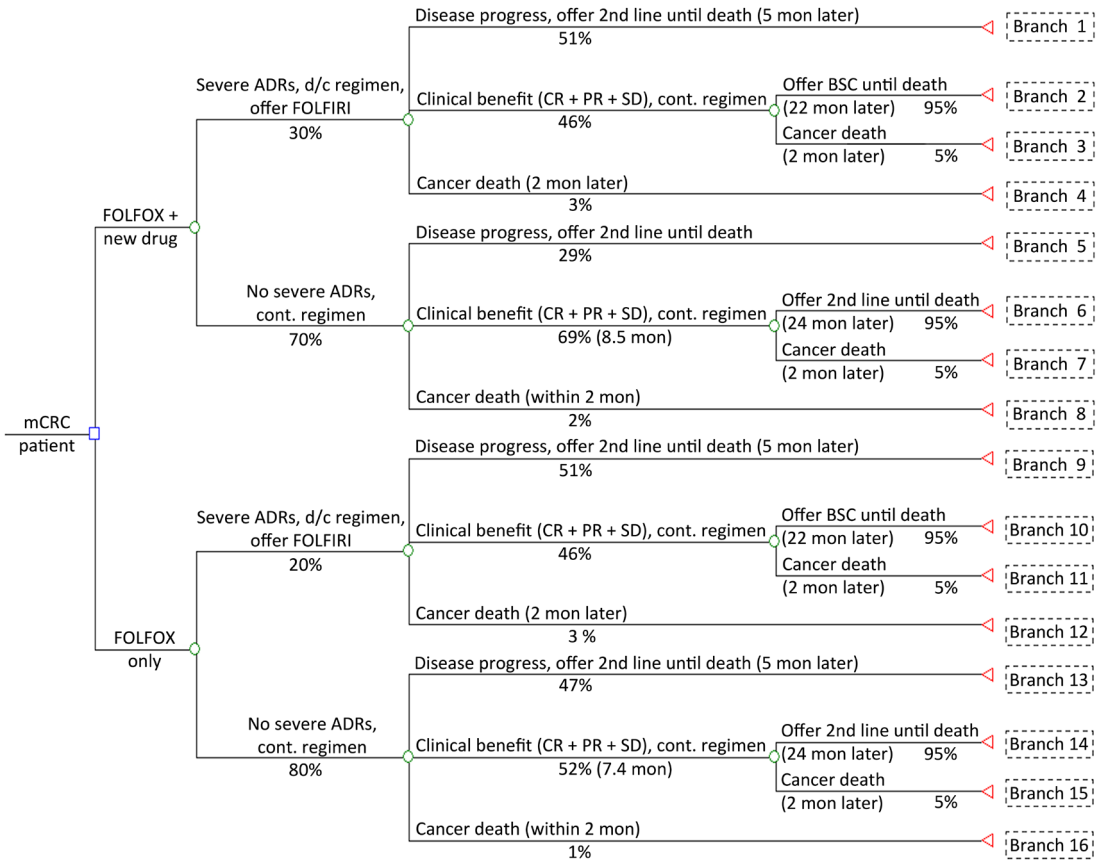


Figure 1: Decision analysis model for the treatment of metastatic colorectal cancer. Abbreviations: mCRC = metastatic colorectal cancer, FOLFOX = oxaliplatin in combination with infusional 5-fluorouracil, FOLFIRI = irinotecan in combination with infusional 5-fluorouracil, ADR = adverse drug reaction, CR =complete response , PR = partial response, SD = stable disease, BSC = best supportive care, mon = month, d/c = discontinued, cont. = continue.

Clinical data

The clinical data required to populate the model consisted of early treatment discontinuations because of toxicity, achievement of clinical benefit (CR, PR, or SD), duration of clinical benefit, risk of cancer-related death during active treatment, and the number of chemotherapy cycles administered. These data were obtained through a literature search of randomised trials evaluating FOLFOX (± bevacizumab) and FOLFIRI in first- and second-line settings, respectively, for the treatment of mCRC. Two randomised trials that provided the required data for the decision model were identified (Table 1). The first trial evaluated FOLFOX or a clinically similar regimen of XELOX (capecitabine plus oxaliplatin) ± bevacizumab in the first-line treatment of mCRC (11). A total of 1401 patients were randomised to receive FOLFOX/XELOX + bevacizumab (n = 699) or FOLFOX/XELOX + placebo (n = 701). The interaction between FOLFOX and XELOX on the primary clinical endpoint was not statistically significant (P = 0.70), thereby justifying the decision to combine patients who received FOLFOX and XELOX. The median progression-free survival was 9.4 months in the bevacizumab group compared with 8.0 months in the placebo group (HR = 0.83, P = 0.023), resulting in a 1.4-month survival benefit (11). Overall, 30% of patients in the bevacizumab group, compared with 20% of the controls, required permanent discontinuation of treatment due to adverse events. Approximately 2% and 1% of patients died during treatment with bevacizumab and placebo, respectively (Table 1).

Data on the safety and efficacy of second-line FOLFIRI following first-line FOLFOX were

Table1: Published randomised trials providing clinical data to populate the economic model

Reference	Treatment arms	Clinical outcomes	
Saltz et al. (11)	FOLFOX/XELOX + bevacizumab	Disease progression	= 29%
		Median PFS	= 9.40 months
		Median duration of response	= 8.45 months
		Overall survival	= 21.30 months
		Treatment discontinuations	= 30%
		Death during treatment	= 2%
		Serious side effects (grade III/IV)	= 16%
		Specific Grade III/IV Side Effects	
		Deep vein thrombosis	= 8%
		Diarrhoea	= 18%
		Bleeding	= 2%
		Neutropenia	= 50%
	FOLFOX/XELOX + placebo	Disease progression	= 47%
		Median PFS	= 8.0 months
		Median duration of response	= 7.4 months
		Overall survival	= 19.9 months
		Treatment discontinuations	= 20%
		Death during treatment	= 1%
		Serious side effects (grade III/IV)	= 8%
		Specific Grade III/IV Side Effects	
		Deep vein thrombosis	= 5%
		Diarrhoea	= 11%
		Bleeding	= 1%
		Neutropenia	= 44%
Tournigand et al. (16)	Second-line FOLFIRI	Disease progression	= 51%
		Death during treatment	= 3%
		Median PFS	= 10.9 months
		Median number of cycles	= 6

Abbreviations: PFS = progression-free survival, OS = overall survival, FOLFOX = oxaliplatin in combination with infusional 5-fluorouracil, FOLFIRI = irinotecan in combination with infusional 5-fluorouracil.

obtained from a randomised sequential trial reported by Tournigand (16). Patients were randomised to receive sequential FOLFOX followed by FOLFIRI or the reverse sequence upon progression. There was no significant difference in progression-free and overall survival (21.5 months in FOLFOX–FOLFIRI versus 20.6 months in FOLFIRI–FOLFOX, P = 0.99) between the 2 sequences (16). Using second-line FOLFIRI, 51% of patients experienced an overall progression-free survival of 2.5 months (16). Approximately 3% of patients died within the first 60 days of second-line FOLFIRI (Table 1).

Estimation of treatment costs

Malaysia's healthcare system is composed of public and private sectors. Physicians are required to complete 3 years of service in public hospitals throughout the nation, ensuring adequate coverage for the general population. With respect to drug access, patients treated under the private system typically have access to a greater selection of therapies than those managed under the public system. However, drug prices and costs for hospital resources tend to be higher in private than in public hospitals. As a result, an analysis was performed for patients treated under the public and private systems.

The duration of the investigation was from the start of first- and second-line sequential chemotherapy until death. Data regarding health care resources and costs for anticancer drugs, materials, patient monitoring, and other related hospital resources (e.g., laboratory, diagnostic tests, and best supportive care) were obtained from 2 private and 2 public health care institutions, using a standardised data collection form. The costs were in Malaysian Ringgit (RM) and converted to US Dollar per currency conversion rates in 2010 (conversion factor $1.00 = RM3.20, as of September 2010).

Patient preferences for alternative health states

The QALY is a way of measuring the impact of disease on a patient. The QALY includes both the quality and the quantity of life lived by a patient, and it is calculated by multiplying the survival gain by the overall utility benefit of one therapy over another. The health-related quality of life (QOL) values measured in the analysis were patient preferences for alternative health outcomes, as depicted in the decision analysis model. In the current study, quality-adjusted progression-free periods were measured as "healthy month equivalents" for the time spent in each outcome of the decision model using the time trade-off (TTO) technique (19). The scores, in months, were then converted to utility measures between 0 and 1, where 0 represented death and 1 represented a state of perfect health or optimal QOL.

The TTO technique is a preference-based approach designed to measure the preferences and QOL of respondents for alternative health states (19). After background information on a particular health state (e.g., a cancer that is not responding to treatment) and the duration within that state are presented, respondents are asked to trade length of life in the poorer health state for a lesser duration in a state of optimal health and QOL. For example, a respondent may prefer to live 4 months of optimal health rather than 12 months confined to a wheelchair. Under this scenario, the utility associated with being in a wheelchair for 12 months would be 0.33 (i.e., 4/12) on a scale between 0 and 1, where 0 represents death and 1 is a state of optimal quality of life. In the economic model, all of the possible outcomes were evaluated with this method and used to weigh the time spent in each health state in terms of QOL.

Intuitively, the ideal population for measuring health state utilities and treatment preferences is cancer patients who are in a position to undergo the new treatment. However, the Canadian Guidelines for Economic Evaluations and the Panel on Cost-effectiveness in Health and Medicine in the United States recommend that treatment preferences should be measured by members of the general public who are potential candidates for the new medical intervention (5,20). As a compromise, in this study, a patient surrogate group was used to provide insight from both the perspective of the patient and members of the general public, as the latter group often has difficulty in understanding utility questionnaires. There is evidence in the oncology literature suggesting that nurses are suitable patient surrogates for objective outcomes and that derived utility estimates do not substantially alter the findings of cost-utility studies (21,22). Therefore, a convenience sample consisting of 24 oncology nurses provided utility values for the model. Using a sample of 24 respondents, healthy month equivalents were measured with a precision of approximately 1.0 month and a 95% probability.

After informed consent was obtained, each participant was interviewed for 30 to 45 minutes by trained local field investigators. Respondents were presented with information on FOLFOX, bevacizumab, and FOLFIRI regarding the methods of administration, their efficacy, and their side effects as reported in the literature. Bevacizumab

was not identified by name, but simply referred to as the "new drug". The interview was continued with a description of 16 health states and the length of time a patient would live in each health state (Figure 1). The respondents were asked how many months of optimal health they considered equivalent to the time spent in each of the less than optimal health states described in the model. These measures were then used to weigh each branch of the model by the QOL experienced by a patient living through that period. An identical process was used for each of the 16 outcomes (Figure 1). The mean healthy month equivalent score for each outcome was then divided by 12 months to estimate the number of QALYs associated with that health state.

A standardised questionnaire supported by printed interview tools and graphical displays was used to facilitate participant understanding of the TTO technique. To minimise the framing effect, all pathways were presented pictorially in a consistent manner. Demographic data were collected from each participant, including years of oncology and colorectal cancer experience, involvement in the development of systemic treatment guidelines for colorectal cancer, familiarity with the cost of anticancer drugs, and family history of colorectal cancer.

Cost-utility analysis

The clinical, economic, and respondents' preference data were combined into a cost-utility analysis of bevacizumab to identify a price per dose that would be considered cost-effective according to the WHO criteria (8,9). The base case analysis assumed that the addition of bevacizumab to standard chemotherapy would provide a survival benefit of 1.4 months. The primary objective of the analysis was to estimate an appropriate price for the bevacizumab with the target benchmark cost of $44 400 per QALY gained, which is 3 times the 2010 Malaysian per capita GDP. Indirect costs were not included because there was no data available on the association between bevacizumab usage and indirect-cost avoidance. Future costs and benefits were not discounted because of the short period involved. However, the stability of the baseline results was evaluated by a comprehensive sensitivity analysis, consisting of substituting the 95% confidence intervals (CIs) for the health state utilities as well as variations in the overall survival benefit, costs of care, and the target threshold for economic value in Malaysia. Costs of care were varied by approximately 15% to include any potential differences across the country. Individual analyses were conducted for patients treated in public and private hospitals.

Results

Clinical outcomes data and costs used to populate the model are presented in Tables 1 and 2. The economic data revealed that the expenses for chemotherapy, the management of side effects, and the best supportive care were lower in the public health care system compared with the private system in Malaysia. This may be a reflection of a slightly lower level of care offered to patients in public hospitals and the ability of the private sector to mark up the cost of goods and health services.

The second component required for the cost-utility analysis was the health state utilities for the time spent in each of the 16 health states (Figure 1). Utilities for each outcome were estimated from a sample of 24 oncology nurses who consented to participate in the study: 14 from public hospitals and 10 from private institutions. The group had an average of 3.4 years of direct oncology experience (ranged 2–8 years), and all had experience in the treatment of colorectal cancer patients. In addition, all respondents had direct clinical experience in the administration of and the follow-up care associated with FOLFOX (mean experience of 2.2 years), and 92% had experience with FOLFIRI chemotherapy (mean experience of 1.9 years). Furthermore, 22 out of 24 (92%) had experience with the newer targeted therapies bevacizumab and cetuximab. Because lack of knowledge about the cost of drugs could affect treatment preferences, respondents were asked to state their knowledge of costs for modern oncology drugs. The findings revealed that 100% were very or somewhat familiar with the cost of drugs used to treat cancer. The final series of demographic questions focused on the respondents' family history of colorectal cancer. The data revealed that none of the 24 subjects had a family history positive for colorectal cancer.

The health state utilities from the oncology nurses are presented in Table 3. The results suggest that patient utilities were influenced by the severity of drug toxicity, the likelihood of achieving a response to chemotherapy, and the risk of rapid cancer death. The health states with the lowest utilities (i.e., branches 11 and 16 of the model in Figure 1) were those when first-line therapy had to be stopped because of severe toxicity and when the patient had an early progression during second-line treatment followed by rapid death due to cancer. It was interesting to note that, in all of the related scenarios, comparative branches that included treatment with the "new drug" tended to have lower health state utilities (Table 3).

Table 2: Hospital costs for the treatment of metastatic colorectal cancer in Malaysia

Recourse item	Public hospitals	Private hospitals
FOLFOX chemotherapy ($ per cycle) [a]	998.00	1047.00
FOLFIRI chemotherapy ($ per cycle) [a]	1395.00	1489 .00
Permanent chemotherapy discontinuation because of toxicity ($) [b]	111.60	241.80
Administration of the "new drug" after FOLFOX chemotherapy ($)	18.60	40.30
Best supportive care ($ per month) [c]	156.00	338.00

[a] Cost per cycle includes resources for drug administration and routine patient monitoring. In the hospitals that provided data for this study, patients are admitted for 2 days to receive chemotherapy.
[b] Patients were admitted for 3 days for the management of side effects and for reassessment.
[c] After failing 2 lines of chemotherapy, patients would receive best supportive care on an outpatient basis until death.
Abbreviations: FOLFOX = oxaliplatin in combination with infusional 5-fluorouracil, FOLFIRI = irinotecan in combination with infusional 5-fluorouracil.

This is likely related to the additional side effects that occur with the addition of an anti-VEGF agent such as bevacizumab to chemotherapy (Table 1).

Cost-utility analysis for public and private hospital systems

The outcomes data from the clinical trial, the estimated costs associated with each treatment, and the health state utility estimates were combined into the cost-utility analysis. The price for 1 dose of bevacizumab was varied until the incremental cost-effectiveness ratio reached a threshold of $44 400 per QALY gained. When using this approach from the public health care system perspective, the base case analysis suggested that a cost per dose that would achieve cost-effectiveness according to the WHO criteria could not be reached because bevacizumab simply did not provide enough of a survival benefit in mCRC patients (Table 4). Similar results were also identified when the analysis was undertaken from the perspective of private hospitals.

A series of one-way sensitivity analyses were conducted using the upper 95% CI for the health state utilities, variations in treatment costs, and the targeted cost per QALY threshold. Identical results in the base-case analysis for both public and private hospitals were achieved. A price per dose that would make bevacizumab cost-effective could not be realised. This was primarily driven by the modest survival benefit offered by bevacizumab in mCRC patients.

The only situation where a cost-effective price per dose was identified occurred when the survival gain was increased to 3 and 6 months. When the survival benefit of bevacizumab was increased from 1.4 to 3 months, the cost per dose for public and private hospitals was estimated to be $567 and $490, respectively. When the survival gain was increased to 6 months, the price per dose of bevacizumab increased further to $1258 and $1182 for public and private institutions, respectively, and these were considered cost-effective according to the WHO criteria (8,9). Therefore, the single biggest factor controlling the cost-effectiveness of bevacizumab is the ability of the drug to increase overall survival.

Bevacizumab is available in Malaysia for a purchase price of approximately $1800 per dose (5 mg/kg) for an average 60-kg mCRC patient. A sensitivity analysis was conducted, and the current price of bevacizumab was applied to the model. The results revealed that the incremental cost per QALY gained would be greater than $200 000 for both public and private institutions. When a $50 000 cost per QALY threshold was used instead of the WHO criteria, a cost-effective price per dose was still not achievable. In summary, the sensitivity analyses suggested that bevacizumab is not a cost-effective drug in Malaysia according to the WHO criteria. To achieve cost-effectiveness, drug performance in terms of survival gain in mCRC patients would need to improve and the price would have to be reduced to between $500 and $1300.

Table 3: Health state utilities derived using the time trade-off technique

Health outcomes evaluated in the decision model	Time in health state (months) [a]	Utility estimate (mean [95% CI]) [b]
FOLFOX ± "new drug" → FOLFIRI → BSC until death		
Branch #1 FOLFOX + "new drug" were discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI for 4 cycles. There was disease progression. The patient received BSC and died 6 months later.	10	0.74 (0.65–0.83)
Branch #2 FOLFOX + "new drug" were discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI. There was a response to FOLFIRI, and the patient went on to receive 8 cycles. Upon progression, the patient received BSC and died 22 months later.	28	0.80 (0.73–0.87)
Branch #3 FOLFOX + "new drug" were discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI. There was a response to FOLFIRI, and the patient went on to receive 8 cycles. Upon progression, the patient received BSC and died 2 months later.	8	0.67 (0.61–0.73)
Branch #4 FOLFOX + "new drug" were discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI for 2 cycles. However, the patient died due to cancer progression within the first 2 months.	4	0.74 (0.65–0.84)
Branch #5 The patient tolerated side effects but had disease progression after 4 cycles of FOLFOX + the "new drug". The patient was then treated with FOLFIRI for 4 cycles, but the disease did not respond. The patient received BSC and died 2 months later.	6	0.82 (0.76–0.89)
Branch #6 The patient tolerated side effects and responded to FOLFOX + "new drug". The patient went on to receive 17 cycles of first-line therapy. Upon progression, the patient went on to receive 6 cycles of FOLFIRI. Upon progression, the patient received BSC and died 21 months later.	29	0.81 (0.77–0.86)
Branch #7 The patient tolerated side effects and responded to FOLFOX + "new drug". The patient went on to receive 17 cycles of first-line therapy. Upon progression, the patient went on to receive 2 cycles of FOLFIRI but died 2 months later.	11	0.83 (0.79–0.87)
Branch #8 The patient tolerated side effects but had disease progression after 2 cycles of FOLFOX + "new drug". The patient died due to cancer 1 month later.	2	0.75 (0.63–0.86)

Health outcomes evaluated in the decision model	Time in health state (months) [a]	Utility estimate (mean [95% CI]) [b]
FOLFOX → FOLFIRI → BSC until death		
Branch #9 FOLFOX was discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI for 4 cycles. There was disease progression. The patient received BSC and died 6 months later.	10	0.82 (0.75–0.82)
Branch #10 FOLFOX was discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI. There was a response to FOLFIRI, and the patient went on to receive 8 cycles. Upon progression, the patient received BSC and died 22 months later.	28	0.81 (0.76–0.86)
Branch #11 FOLFOX was discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI. There was a response to FOLFIRI, and the patient went on to receive 8 cycles. Upon progression, the patient received BSC and died 2 months later.	8	0.72 (0.66–0.79)
Branch #12 FOLFOX was discontinued after 2 cycles due to side effects, and the patient was then treated with FOLFIRI for 2 cycles. However, the patient died due to cancer progression within the first 2 months.	4	0.75 (0.66–0.84)
Branch #13 The patient tolerated side effects but had disease progression after 4 cycles of FOLFOX. The patient was then treated with FOLFIRI for 4 cycles, but the disease did not respond. The patient received BSC and died 2 months later.	6	0.84 (0.76–0.92)
Branch #14 The patient tolerated side effects and responded to FOLFOX. The patient went on to receive 15 cycles of first-line therapy. Upon progression, the patient went on to receive 6 cycles of FOLFIRI. Upon progression, the patient was offered BSC and died 21 months later.	32	0.91 (0.88–0.94)
Branch #15 The patient tolerated side effects and responded to FOLFOX. The patient went on to receive 15 cycles of first-line therapy. Upon progression, the patient went on to receive 2 cycles of FOLFIRI but died 2 months later.	11	0.84 (0.79–0.90)
Branch #16 The patient tolerated side effects and but had disease progression after 2 cycles of FOLFOX. The patient died due to cancer progression 1 month later.	2	0.75 (0.63–0.86)

[a] As presented in each branch of the decision model.
[b] A quality of life score for a health state between 0 and 1, with 0 = death and 1 = optimal health.
Abbreviations: FOLFOX = oxaliplatin in combination with infusional 5-fluorouracil, FOLFIRI = irinotecan in combination with infusional 5-fluorouracil, BSC = best supportive care.

Table 4: Sensitivity analysis on the cost per dose of the "new drug"

Sensitivity analysis [a]	Public hospitals	Private hospitals
Base case	Not reached	Not reached
Upper 95% CI of health state utilities for chemotherapy + "new drug"	Not reached	Not reached
Changing cost of FOLFOX chemotherapy by ± 15%	Not reached	Not reached
Changing cost of FOLFIRI chemotherapy by ± 15%	Not reached	Not reached
Changing cost of BSC cost by ± 15%	Not reached	Not reached
Changing cost of ADR cost by ± 15%	Not reached	Not reached
Changing survival benefit of the "new drug" from 1.4 to 3 months	$567	$490
Changing survival benefit of the "new drug" from 1.4 to 6 months	$1258	$1182
Using the current cost of bevacizumab ($1800 per dose) in Malaysia	Not cost-effective	Not cost-effective
Setting the threshold for cost effectiveness at $50 000 per QALY gained	Not reached	Not reached

[a] For a target threshold of US $44 400 per QALY when the new drug is added to FOLFOX chemotherapy.
Abbreviations: FOLFOX = oxaliplatin in combination with infusional 5-fluorouracil, FOLFIRI = irinotecan in combination with infusional 5-fluorouracil, BSC = best supportive care, ADR = adverse drug reaction costs.

Discussion

Decision analysis modelling is a powerful simulation technique widely used to perform cost-effectiveness evaluations of new drugs. In such studies, the health services researcher develops a decision model comparing the new therapy to the current standard, incorporates the costs and consequences of the two alternatives into the analysis, and estimates the incremental cost per QALY gained using the new intervention. If the cost per QALY is below a pre-determined threshold, the conclusion is that the new treatment is cost-effective and should be added to a hospital or a national formulary.

Decision analysis is a useful tool that can also be used to estimate any unknown in the analysis. The unknown in most published studies has been the incremental cost per QALY gained. However, decision analysis can also be applied in the context of pricing a new drug before it is introduced to the market. In this study, the latter process was used to estimate the cost of bevacizumab, a drug that provides a 1.4-month survival benefit when added to chemotherapy in the first-line treatment of mCRC (11).

The analysis was conducted from the perspective of both the Malaysian public and private health care systems using the WHO criteria for cost-effectiveness. In the base case analysis and in most of the scenarios evaluated, a cost per dose resulting in cost-effectiveness could not be identified because a 1.4-month survival gain was inadequate. A cost-efficient price was only realised when the survival gain from bevacizumab was artificially increased to at least 3 months. When the current Malaysian price per dose (i.e., $1800 for bevacizumab was evaluated, the drug was not considered to be cost-effective according to the WHO criteria.

The findings of this study suggest that the WHO criteria for cost-effectiveness can be applied to a country such as Malaysia for estimating an appropriate price that may be more affordable to the national health care system. Furthermore, our results suggest that bevacizumab is priced excessively high in Malaysia considering the 1.4-month survival benefit that it provides to mCRC patients. For the drug to become cost-effective, the price would have to be reduced and a new treatment algorithm that would increase survival to at least 3 months would need to be identified.

There are a number of limitations in the application of this technique that need to be addressed. Given the lack of data for each cycle of chemotherapy, we constructed a decision tree instead of a Markov model to simulate the clinical and economic consequences of chemotherapy for patients with mCRC; the latter would have been preferable given its ability to incorporate the element of time. For the proposed methodology to be viable, complete data from randomised trials on a drug-by-drug basis is required. This is not always possible. One of the limitations of using the per capita GDP for value-based pricing is

that it represents a national average and does not consider income dispersion. Our study measured health state utilities from a sample of oncology nurses. However, the external validity of our findings would have been enhanced if we had also included patients, family members, and members of the general public. For our modelling strategy to be applied, a new drug must demonstrate either an improvement in QOL over the standard of care or a survival of sufficient magnitude to identify a final price point for cost-effectiveness. In the case of bevacizumab, the drug simply did not provide enough of a survival benefit to identify a price that would be considered cost-effective. Lastly, indirect costs, such as time off work, may be relevant in this setting but were not considered in this analysis because there was a lack of such data in the mCRC literature. Future modelling should consider these elements.

Conclusion

The current paper presents a systematic process to estimate drug costs based on pre-determined thresholds for societal value. The advantages of this technique are that it is relatively straightforward to perform, that it is transparent, and that the decision model can be easily applied to other jurisdictions using local cost data. This information is of value to drug manufacturers and formulary committees because it facilitates negotiations for optimal pricing in a given jurisdiction.

Authors' Contributions

Conception and design, analysis and interpretation of the data: GD, IT, MSL
Provision of study materials: NNS, VMM, SBM
Statistical expertise, drafting of the article: GD
Critical revision and final approval of the article: IT, MSL, NNS, VMM, SBM

Correspondence

Mr George Dranitsaris
BPharm (University of Toronto),
MS (University of Toronto)
283 Danforth Ave, Suite 448
Toronto, Ontario, M4K 1N2
Canada
Tel: +1 (416) 461-2720
Fax: +1 (416) 461-4735
Email: gdranit@ca.inter.net

References

1. Orszag PR, Ellis P. The challenge of rising health care costs—A view from the Congressional Budget Office. *N Engl J Med*. 2007;**357(18)**:1793–1795.

2. Hoffman JM, Shah ND, Vermeulen LC, Doloresco F, Martin PK, Blake S, et al. Projecting future drug expenditures—2009. *Am J Health Syst Pharm*. 2009;**66(3)**:237–257.

3. Arenas-Guzman R, Tosti A, Hay R, Haneke E. Pharmacoeconomics—An aid to better decision-making. *J Eur Acad Dermatol Venereol*. 2009; **19 Suppl 1**:34–39.

4. *Guidelines for the economic evaluation of health technologies: Canada* [Internet]. 3rd ed. Ottawa (CA): Canadian Agency for Drugs and Technologies in Health; 2006 [cited 2009 Nov 18]. Available from: http://www.cadth.ca/media/pdf/186_EconomicGuidelines_e.pdf.

5. Weinstein MC, Fineberg HV. *Clinical decision analysis*. Philadephia (PA): WB Saunders; 1980.

6. Drummond MF, Mason AR. European perspective on the costs and cost-effectiveness of cancer therapies. *J Clin Oncol*. 2007;**25(2)**:191–195.

7. Wilking N, Jonsson B. *A Pan European comparison regarding patient access to cancer drugs*. Stockholm (SE): Karolinska Institutet, Stockholm School of Economics; 2005.

8. Hillner BE, Smith TJ. Efficacy does not necessarily translate to cost effectiveness: A case study in the challenges associated with 21st-century cancer drug pricing. *J Clin Oncol*. 2009;**27(13)**:2111–2113.

9. Murray CJ, Evans DB, Acharya A, Baltussen RM. Development of WHO guidelines in generalized cost-effectiveness analysis. *Health Econ*. 2000;**9(3)**: 235–251.

10. *The world factbook* [Internet]. Washington (DC): Central Intelligence Agency; 2009 [cited 2010 Sep 18]. Available from: https://www.cia.gov/library/publications/the-world-factbook/rankorder/2004rank.html.

11. Saltz LB, Clarke S, Diaz-Rubio E, Scheithauer W, Figer A, Wong R, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: A randomized phase III study. *J Clin Oncol*. 2008;**26(12)**:2013–2019.

12. Shiroiwa T, Fukuda T, Tsutani K. Cost-effectiveness analysis of bevacizumab combined with chemotherapy for the treatment of metastatic colorectal cancer in Japan. *Clin Ther*. 2007;**29(10)**:2256–2267.

13. Tappenden P, Jones R, Paisley S, Carroll C. Systematic review and economic evaluation of bevacizumab and cetuximab for the treatment of metastatic colorectal cancer. *Health Technol Assess*. 2007;**11(12)**:1–128, iii–iv.

14. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: A multicentre randomized trial. *Lancet*. 2000;**355(9209)**:1041–1047.

15. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol*. 2004;**22(1)**:23–30.

16. Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: A randomized GERCOR study. *J Clin Oncol*. 2004;**22(2)**:229–237.

17. Engstrom PF. Systemic therapy for advanced or metastatic colorectal cancer: National Comprehensive Cancer Network guidelines for combining anti-vascular endothelial growth factor and anti-epidermal growth factor receptor monoclonal antibodies with chemotherapy. *Pharmacotherapy*. 2008; **28(11 Pt 2)**:18S–22S.18.

18. Sonnenberg FA, Beck RJ. Markov models in medical decision making: A practical guide. *Med Decis Making*. 1993;**13(4)**:322–332.

19. Torrance GW. Utility approach to measuring health-related quality of life. *J Chron Dis*. 1987;**40(6)**: 593–603.

20. Russell LB, Gold MR, Siegel JE, Daniels N, Weinstein MC. The role of cost-effectiveness analysis in health and medicine. Panel on Cost-Effectiveness in Health and Medicine. *JAMA*. 1996;**276(14)**:1172–1177.

21. Leung P, Tanock IF, Oza AM, Puodziunas A, Dranitsaris G. Cost-utility analysis of chemotherapy using paclitaxel, docetaxel, or vinorelbine for patients with anthracycline-resistant breast cancer. *J Clin Oncol*. 1999;**17(10)**:3082–3090.

22. Ortega A, Dranitsaris G, Sturgeon J, Sutherland H, Oza A. Cost-utility analysis of paclitaxel in combination with cisplatin for patients with advanced ovarian cancer. *Gynecol Oncol*. 1997;**66(3)**:454–463.

Original Article

A Nested Allele-Specific Multiplex Polymerase Chain Reaction Method for the Detection of *DRD2* Polymorphisms

Zalina Zahari[1,2], Mohd Razali Salleh[3], Mohd Khairi Zahri @ Johari[4], Nurfadhlina Musa[5], Rusli Ismail[5]

Submitted: 27 Mar 2011
Accepted: 1 Jun 2011

[1] *Pharmacogenetics Research Group, Institute for Research in Molecular Medicine, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia*

[2] *Department of Pharmacy, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[3] *Department of Psychiatry, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia*

[4] *Faculty of Medicine and Health Sciences, Universiti Sultan Zainal Abidin City Campus, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia*

[5] *Institute for Research in Molecular Medicine, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia*

Abstract

Background: **The dopamine D2 receptor gene (*DRD2*) plays a role in many diseases such as schizophrenia, Parkinson's disease, and addictive behaviour. Methods currently available for the detection of *DRD2* polymorphisms are costly and cannot detect all 8 polymorphisms of our research interest simultaneously (Val96Ala, Leu141Leu, Val154Ile, Pro310Ser, Ser311Cys, *TaqI* A, *A*-241*G*, and −141C *Ins/Del*). Therefore, we developed a nested multiplex polymerase chain reaction (PCR) for simultaneous detection of these polymorphisms.**

Methods: **Genomic DNA was extracted from blood using standardised methods. Primers specific at the 3'-end for the polymorphic sites were designed. A two-step PCR method was developed. In the first PCR, a region from exon 3 to 4, exon 7, the promoter region, and the 3'-region of *DRD2* were specifically amplified. The products were subsequently used as templates in the second PCR. Sequencing was performed to validate the test results.**

Results: **Specific bands corresponding to the amplified product of interest were obtained. The method was reproducible and specific when used to genotype patients with schizophrenia. The amplified sequences showed 100% homology to the *DRD2* sequence.**

Conclusion: **The method was found to be simple, rapid, specific, and reproducible for the simultaneous detection of the *DRD2* polymorphisms.**

Keywords: *dopamine D2 receptor, genetics, genetic polymorphism, methods, nested PCR, reproducibility of results, specificity*

Introduction

The dopamine D2 receptor (DRD2) belongs to the G protein–coupled receptor superfamily located on postsynaptic dopaminergic neurons (1). It is mainly expressed in the striatum, cortex, and limbic system (2). The human DRD2 gene is located at 11 q22-q23 (3), as depicted in Figure 1. This gene was previously found to contain 8 exons that span over 270 kb (4), but it has recently been found to span over only 65.69 kb (GenBank accession number NG_00884.1). Of the more than 200 polymorphisms at the *DRD2* locus that may have important clinical and physiological implications, the most important polymorphisms are Val96Ala, Leu141Leu, Val154Ile, Pro310Ser, Ser311Cys, DRD2/ANKK1 *TaqI* A (referred to here as *TaqI* A), *A*-241*G*, and −141C *Ins/Del* (3,5–10), as detailed in Table 1.

The *TaqI* A1 allele of the *TaqI* A polymorphism is associated with reduced DRD2 density (9,10). The *TaqI* A1 allele has been shown to be associated with addictive behaviour, including alcoholism (11) and smoking (12). This allele has also been implicated in the development of motor fluctuations in patients with Parkinson's disease in response to levodopa (13). Previous studies have shown that the –141C *Ins/Del* polymorphism leads to reduced promoter activity in vitro (8). The –141C *Del* allele was found to be associated with a high striatal dopamine receptor density in healthy volunteers (10) as well as with schizophrenia (14) and the clinical response to antipsychotics in the first episode of schizophrenia (15).

Previously described methods for the detection of *DRD2* polymorphisms include polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) (5,6,8,16–19), denaturing gradient gel electrophoresis (6), Southern blot analysis (3,16,20), and direct sequencing (3,16,20). These methods are generally costly and tedious, and some require specialised equipment and manpower that may not be available or appropriate for all research groups. The objective of this paper is to describe a novel method for the detection of these 8 *DRD2* polymorphisms that is simple and relatively rapid while maintaining sensitivity. This assay was developed for a bigger study to investigate the influence of *DRD2* polymorphisms on treatment outcomes in patients with schizophrenia. Application of this method will enable smaller research groups to increase the scale of their *DRD2* genotyping throughput by less specialised personnel in less specialised settings.

Materials and Methods

Genomic DNA

Genomic DNA was obtained from peripheral leucocytes extracted from 10 mL of blood taken from patients with schizophrenia attending the psychiatry clinic at Hospital Universiti Sains Malaysia, using previously described methods (21). The protocol for this study was approved by the Research and Ethics Committee, Universiti Sains Malaysia, Kelantan, Malaysia.

Primer design

To improve sensitivity, the PCR was designed as a two-step nested PCR where, in the first PCR, primers were designed to amplify specific regions of the DRD2 gene containing the mutations of interest. The products were then used as templates for the second allele-specific reactions. For the second PCR, primers were designed to have specific 3'-ends, manipulated to differentiate single nucleotide changes at the specific locus during PCR amplifications. We identified the location of the flanking regions of the primers against the DNA sequences published by Hauge et al. (16) and Murakami et al. (22) that are available at http://www.ncbi.nlm.nih.gov/ (accession number: AF050737 and AF148806).

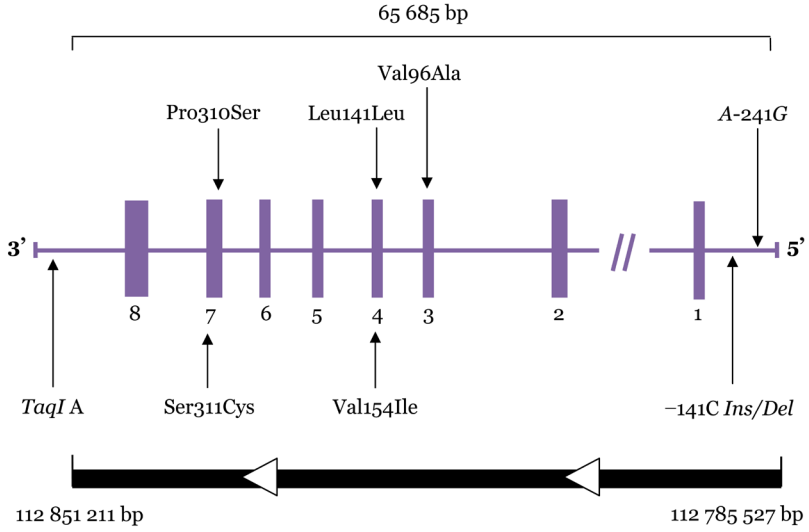


Figure 1: Dopamine D2 receptor gene (*DRD2*) structure and polymorphisms studied. Boxes represent exons; horizontal lines connecting boxes represent introns, promoter, and untranslated regions. Arrows indicate relative locations of the polymorphisms.

Table 1: Characteristics and positions of 8 dopamine D2 receptor polymorphisms

Polymorphism	Characteristic mutation	Position	Accession no.
Val96Ala	Substitution of alanine for valine at codon 96	14858	AF050737
Leu141Leu	Silent substitution at codon 141 (C T)	16019	AF050737
Val154Ile	Substitution of isoleucine for valine at codon 154	16058	AF050737
Pro310Ser	Substitution of serine for proline at codon 310	20225	AF050737
Ser311Cys	Substitution of cysteine for serine at codon 311	20229	AF050737
TaqI A	Alteration of a *TaqI* restriction site at 10 541 kb downstream of *DRD2* stop codon (exon 8)	32806	AF050737
A-241G	Substitution of guanine for adenosine at –241	6091	AF148806
–141C Ins/Del	Single base pair cytosine insertion/deletion at position –141	6191	AF148806

We also used the Basic Local Alignment Search Tool (BLAST) programme at http://www.ncbi.nlm.nih.gov/blast to ascertain the specificity of the primers. The primers were also designed to have similar annealing temperatures and appropriate length as well as GC contents for multiplexing reactions to avoid incompatibility of the primer sets. Table 2 lists the sequences of the primers used for both the first and the second PCRs.

Method development for nested allele-specific multiplex PCR

The schematic chart for the detection of *DRD2* polymorphisms using a nested allele-specific multiplex PCR is shown in Figure 2. A total of 276 bp (fragment A) of the 5'-untranslated region (i.e., promoter region) of *DRD2* was amplified with the primers D2PRFW and D2PRRV. It was then used as template for the second PCR of the *A-241G* and –141C *Ins/Del* polymorphisms. For identification of Val96Ala, Leu141Leu, and Val154Ile polymorphisms, the larger first PCR fragment was amplified by using primers DRD2EX3&4FW and DRD2EX3&4RV spanning exons 3 to 4. Fragment B, as large as 1497 bp, was then used as a template for the second PCR. Primers DRD2EX7FW and DRD2EX7RV were used to amplify exon 7 for fragment C. It was then used as template for the second PCR of Pro310Ser and Ser311Cys. Fragment D spanning the 3'-untranslated region (i.e., *TaqI* A region) of *DRD2* was amplified using primers D2TAQ1AFW and D2TAQ1ARV. It was used as a template for the second PCR of the *TaqI* A polymorphism.

Initially, 4 PCR procedures were performed using singlet pairs of primers to determine a PCR programme that would allow optimal amplifications of all the loci when performed individually. The initial reaction condition was determined empirically. When these initial experiments produced a successful uniplex PCR, a multiplex PCR was attempted first by combining the 4 primer sets in a single reaction. The PCR protocols initially employed were exactly the same as they were with the reactions with individual primer pairs.

To improve the amplification of certain loci while eliminating the amplifications of non-specific products, further adjustments to the PCR protocols were made. The concentrations of $MgCl_2$ and *Taq* polymerase were varied. Different combinations of primer pairs and template dilutions were also tried to improve the reproducibility, specificity, and sensitivity. The same strategies were used for the optimisation of the second PCR.

Optimised PCR method for genotyping DRD2

After numerous experiments, the most robust protocol that also gave equal amplification of all alleles was achieved in a total volume of 25.0 µL, containing 200 ng DNA template, 1.0 mM $MgCl_2$, 0.2 mM dNTPs (Promega, Madison, Wisconsin, USA), 0.5 U Biotool® DNA *Taq* Polymerase (B&M Labs, Madrid, Spain), and 1× Biotool® PCR buffer (B&M Labs, Madrid, Spain). The optimal primer (Invitrogen, California, USA) concentrations were found to be 0.15–0.40 µM (Table 1). All the PCRs were done in standard 0.2 mL Eppendorf PCR tubes and ran in an Eppendorf Mastercycler Gradient® Cycler (Eppendorf, Hamburg, Germany).

The first PCR amplified a region from exon 3 to 4 of *DRD2*, exon 7, the promoter region, and the downstream region of the *DRD2* stop

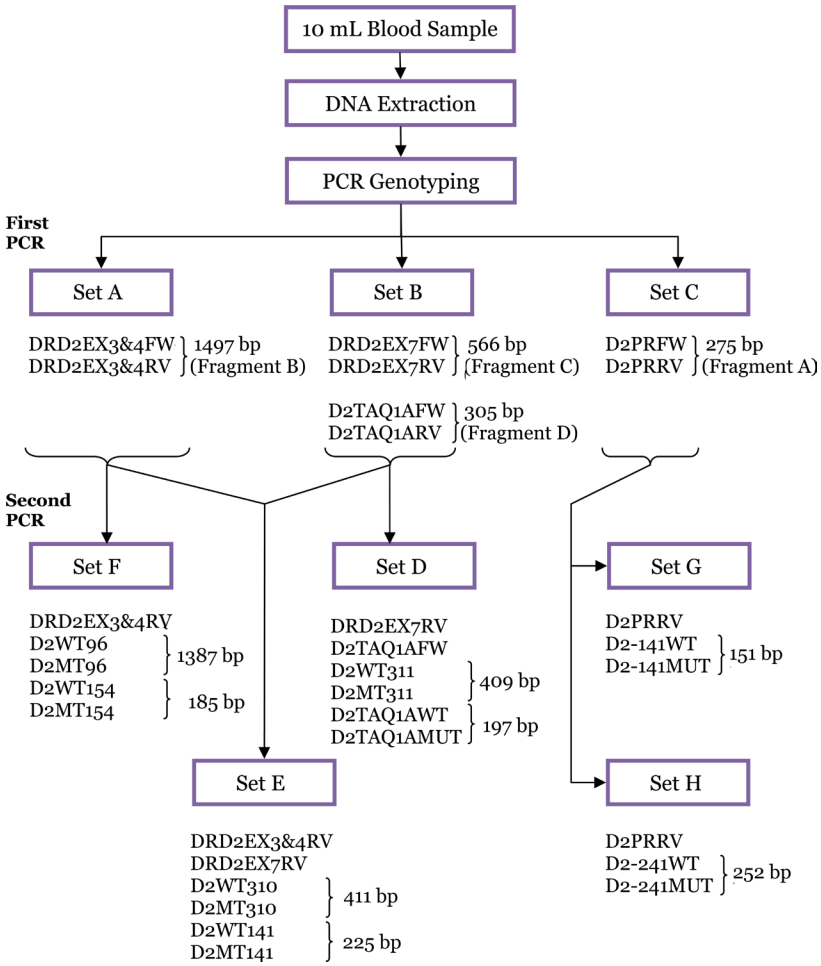


Figure 2: Schematic chart of SNP genotyping of *DRD2* polymorphisms using a nested allele-specific multiplex PCR.

codon (exon 8) using specifically designed primers (Table 2). This step was performed to isolate regions of interest containing the relevant *DRD2* polymorphisms that were later used for the second allele-specific PCR to avoid amplification of similar sequences in the human genome that may be located outside the gene. The protocols involved 2 uniplex and 1 duplex PCRs for improved specificity and sensitivity. The uniplex reactions amplified the region from exon 3 to exon 4 (Set A) and the promoter region (Set C) of *DRD2*, and the duplex reaction amplified exon 7 and the 3'-region (Set B) of the gene. Four primer sets were used in the first PCR, yielding fragments of sizes 1497, 566, 276, and 305 bp. For all the reactions, DNA was denaturated initially at 94 °C for 2 minutes before the cycling programme, followed by 35 cycles of DNA denaturing step at 94 °C for 1 minute, annealing at 65 °C for 1 minute, extension at 72 °C for 2 minutes, and a final extension period at 72 °C for 5 minutes. The PCR products were analysed on a 2.0% agarose gel (LE, analytical grade; Promega, Madison, Wisconsin, USA) stained in ethidium bromide in 1× Tris-borate-EDTA (TBE) buffer at 130 V for 90 minutes.

After a successful first PCR, 2.0 µL of diluted PCR product were used as a template for the detection of wild-type or mutant-type alleles in the second PCR. The second PCR was carried out in a reaction mixture that was identical to that described for the first PCR, with the exception of the primer concentrations shown in Table 2. The second PCR was comprised of 15 cycles of DNA denaturing at 94 °C for 1 minutes, annealing at 63 °C for 1 minutes, and extension at 72 °C for 2 minutes. Next, 10 mL of the second PCR product was analysed on a 2.0% agarose gel and 1× TBE at 130 V for 90 minutes. The expected fragment size for each of the products is listed in Table 2.

Table 2: List of primer sequences, fragment sizes, calculated melting temperature (T_m), and primer concentration of the first and second PCRs used for the detection of 8 dopamine D2 receptor polymorphisms

PCR	Primer	Sequence (5' – 3')	Fragment size (bp)	Calculated T_m (°C)	Primer concentration (µM)
1st PCR					
Set A	DRD2EX3&4FW	cag ctg cct cct gag tct gt	1497	64	0.30
	DRD2EX3&4RV	cca tat ctg tgc cag gga ct		62	0.30
Set B	DRD2EX7FW	ctg atg cct ggg aac ttg tc	566	62	0.15
	DRD2EX7RV	gcc cat ctg taa agt gag ca		60	0.15
	D2TAQ1AFW	acg gct ggc caa gtt gtc t	305	60	0.25
	D2TAQ1ARV	acc ttc ctg agt gtc atc aac		62	0.25
Set C	D2PRFW	act ggc gag cag acg gtg a	276	62	0.40
	D2PRRV	tga agc tgg aca gct ctg c		60	0.40
2nd PCR					
Set D	DRD2EX7RV	cca tat ctg tgc cag gga ct			0.25
	D2TAQ1AFW	acg gct ggc caa gtt gtc t			0.25
	D2WT311	tga ctc tcc ccg acc cgt c	409	60	0.25
	D2MT311	tga ctc tcc ccg acc cgt g		60	0.25
	D2TAQ1AWT	atc ctc aaa gtg ctg gtc g	197	58	0.25
	D2TAQ1AMUT	atc ctc aaa gtg ctg gtc a		56	0.25
Set E	DRD2EX3&4RV	cca tat ctg tgc cag gga ct			0.25
	DRD2EX7RV	gcc cat ctg taa agt gag ca			0.25

PCR	Primer	Sequence (5' – 3')	Fragment size (bp)	Calculated T_m (°C)	Primer concentration (µM)
	D2WT310	gct gac tct ccc cga cc	411	58	0.25
	D2MT310	gct gac tct ccc cga ct		56	0.25
	D2WT141	ctg tgg cca tgc cca tgc	225	60	0.25
	D2MT141	ctg tgg cca tgc cca tgt		58	0.25
Set F	DRD2EX3&4RV	cca tat ctg tgc cag gga ct			0.25
	D2WT96	tgt tgc ttt gtc ccc agg t	1387	58	0.25
	D2MT96	tgt tgc ttt gtc ccc agg c		60	0.25
	D2WT154	caa gcg ccg ggt cac cg	185	60	0.25
	D2MT154	caa gcg ccg ggt cac ca		58	0.25
Set G	D2PRRV	tga agc tgg aca gct ctg c			0.25
	D2-141WT	aac ccc tcc tac ccg ttc c	151	62	0.25
	D2-141MUT	aac ccc tcc tac ccg ttc a		60	0.25
Set H	D2PRRV	tga agc tgg aca gct ctg c			0.25
	D2-241WT	cag cct gca atc aca gct ta	252	60	0.25
	D2-241MUT	cag cct gca atc aca gct tg		62	0.25

The optimised method was validated for reproducibility and specificity. This method was tested against DNA samples obtained from patients with schizophrenia. Samples detected with *DRD2* polymorphisms were identified, re-amplified, and sent for direct sequencing. These were later used as positive controls for the alleles.

Direct DNA sequencing

Specificity of the primer sets used in this study was confirmed using a panel of single PCR products from the first PCR as positive controls that contained either heterozygous or homozygous wild-type/mutant-type, wild-type/wild-type, or mutant-type/mutant-type alleles. Direct sequencing was used to validate each positive

control sample. The DNA samples were purified using QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced on an ABI 3700 using Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA).

Results

Our final method was the result of the optimisation of many factors such as primer pair selection, magnesium amount, *Taq* polymerase amount, and annealing temperatures. In general, the PCR conditions were optimised separately for each polymorphism and then combined using the following steps: (i) design of specific primers, (ii) selection of annealing temperature at which the primers were specific, (iii) determination of magnesium amount and *Taq* polymerase amount, and (iv) different combinations of primer pairs and template dilution.

As shown in Figure 3, the primers designed for the first PCR successfully produced the desired products from the genomic DNA extracted. These were intended as templates for our second allele-specific PCR. The primers designed for the second PCR were also later found to produce the desired second PCR products (Figure 4). Furthermore, the primers designed were found to be compatible with each other with no evidence of misannealing,

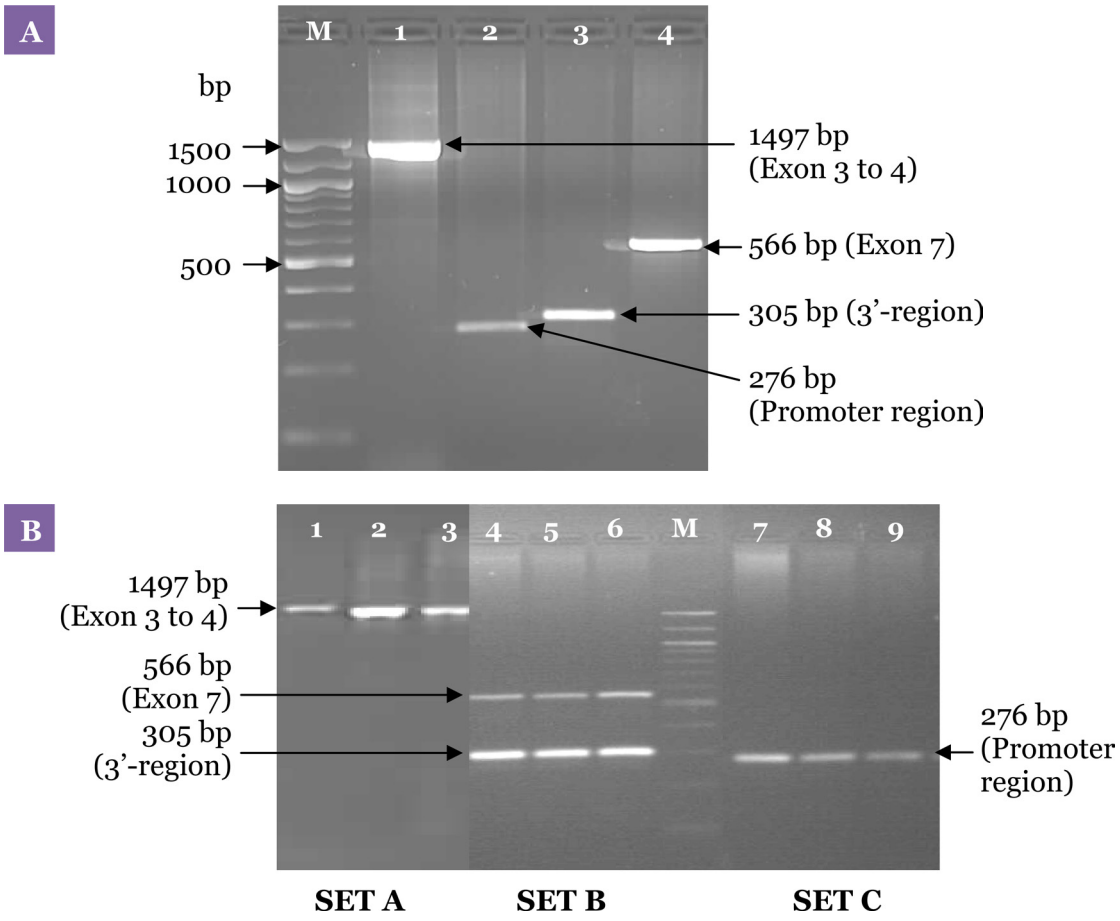
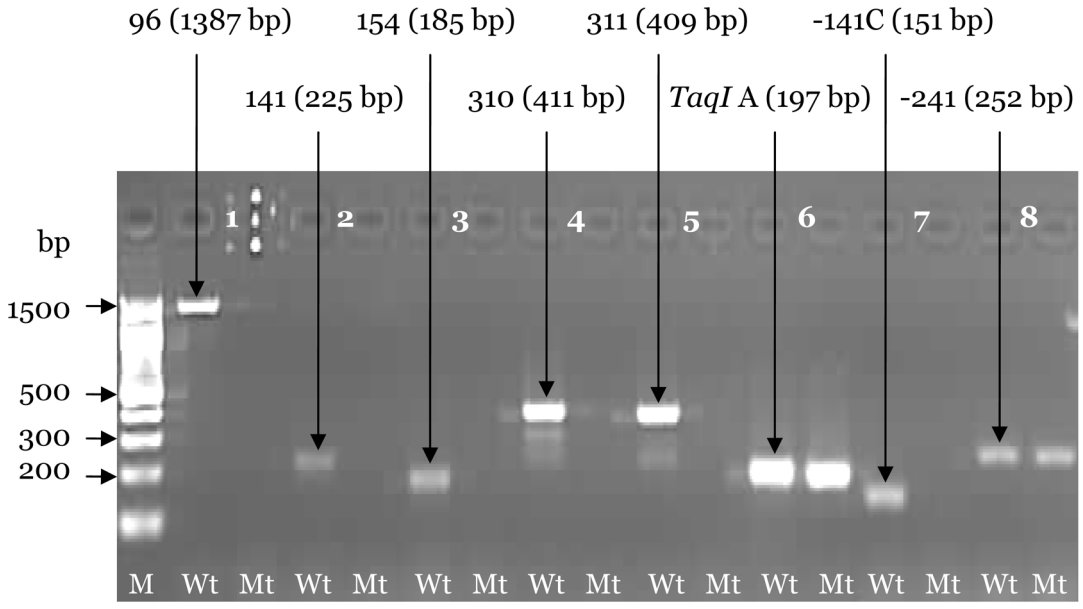


Figure 3: Electrophoresis pattern for the first PCR carried out with the specific primers. (A) Uniplex amplification of the 4 regions of *DRD2*. Lane 1: exon 3 to 4 with primer sets DRD2EX3&4FW and DRD2EX3&4RV; Lane 2: the promoter region (i.e., 5'-untranslated region) with D2PRFW and D2PRRV; Lane 3: the 3'-untranslated region (i.e., *TaqI* A) with D2TAQ1AFW and D2TAQ1ARV; Lane 4: exon 7 with DRD2EX7FW and DRD2EX7RV. (B) Result of the first PCR of 3 DNA samples that were obtained using the optimised PCR method. Lanes 1–3: PCR amplification of Set A, exon 3 to 4; Lanes 4–6: Set B, exon 7 and the 3'-untranslated region simultaneously; Lanes 7–9: Set C, the promoter region. M: 100-bp DNA ladder.

and the primers produced the specific products for both PCRs (Figures 3 and 4).

A uniplex first PCR for each single locus was performed to amplify the region from exon 3 to 4, the promoter region, the 3'-region and exon 7 of *DRD2* with primer sets DRD2EX3&4FW and DRD2EX3&4RV, D2PRFW and D2PRRV, D2TAQ1AFW and D2TAQ1ARV, and DRD2EX7FW and DRD2EX7RV, respectively, as shown in Figure 3A. Subsequently, a multiplex first PCR was performed using exactly the same PCR programme as with individual primer pairs except that the primer sets for all 4 regions of *DRD2* were combined in a single reaction. Optimisation of the multiplex PCR method was unsuccessful due to problems such as non-reproducible amplification of the promoter region and exon 3 to 4. Finally, the problematic primer sets were separated resulting in 2 uniplex and 1 duplex first PCR sets. The methods were further optimised by reducing the amount of magnesium and *Taq* polymerase. The $MgCl_2$ concentration was varied between 2.0 and 1.0 mM. The *Taq* polymerase concentrations used for the assay were varied from 1.0 U to 0.5 U. Figure 3B shows the agarose gel electrophoresis of the first PCR products using the final optimised method.

The second PCR condition was optimised separately for each polymorphism before being combined. Figure 4 shows the result of the uniplex second PCR amplification performed using the same cycling conditions. A multiplex second PCR was developed by combining the desired primer pairs at equimolar concentration in a single reaction. The first parameter that was tested and found to be important was annealing temperature (T_m). The annealing temperature was increased step-wise from 50 °C to 65 °C to eliminate the non-specific products and to avoid false negative results. We found that increasing the annealing temperature decreased the intensity of the amplified non-specific bands.



1	2	3	4	5	6	7	8
96	141	154	310	311	*TaqI*	−141C	−241
Val/Val	Leu C/C	Val/Val	Pro/Pro	Ser/Ser	A2/A1	*Ins/Ins*	*A/G*

Figure 4: Uniplex second PCR products carried out with the specific primers for Val96Ala, Leu141Leu, Val154Ile, Pro310Ser, Ser311Cys, *TaqI* A, −141C *Ins/Del*, and *A*-241*G* polymorphisms. The genotypes of the samples are homozygous wild-type for Val96Ala, Leu141Leu, Val154Ile, Pro310Ser, Ser311Cys, and −141C *Ins/Del* polymorphisms, and heterozygotes for *TaqI* A and *A*-241*G* polymorphisms. M: 100-bp DNA ladder; Wt: wild-type; Mt: mutant-type.

The optimum amount of $MgCl_2$ was 1.0 mM (Figure 5). Although higher concentrations of $MgCl_2$ increased the intensity of the desired bands, it also produced more non-specific backgrounds. A range of *Taq* polymerase concentrations were also tested during the experiments. With 1.0 U of *Taq* polymerase, although all the desired bands were amplified, non-specific bands were also observed. The non-specific bands were successfully eliminated by reducing the amount of *Taq* polymerase to 0.5 U (Figure 5).

Thus, the condition for optimum sensitivity, specificity, and robustness for all the desired polymorphisms for the second PCR involved the use of 1.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U *Taq* polymerase, 2.0 µL (1:100) template, and 0.25 µM of each primer pair (Set D, E, F, G, and H) in a final volume of 25.0 µL at an annealing temperature of 63 °C (Figure 6).

The optimised method was shown to be specific, sensitive, and reproducible when validated against the 156 DNA samples obtained

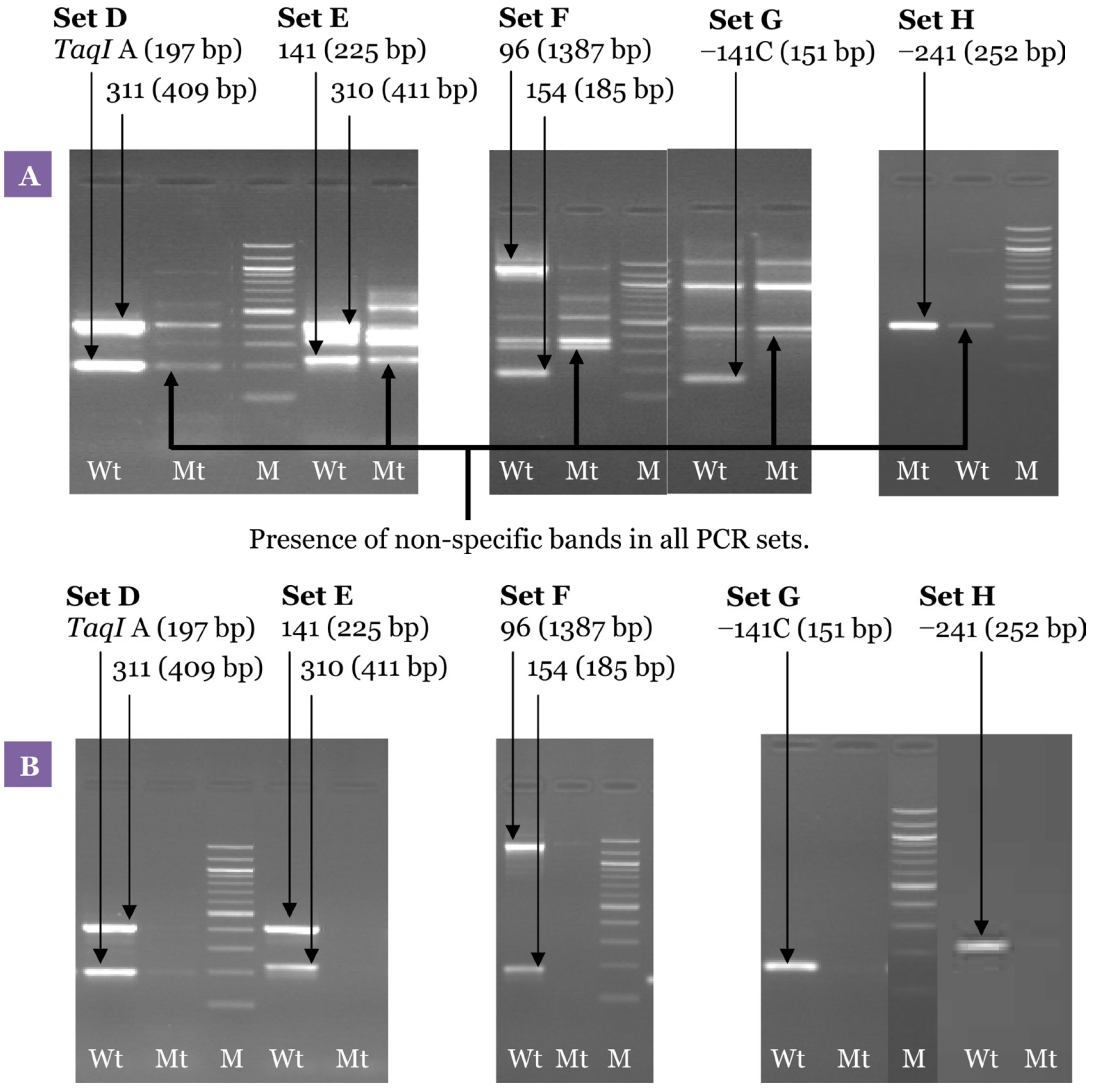


Figure 5: Optimisation of the allele-specific multiplex PCR. Agarose gel electrophoresis of the products from the second PCR showing the effect of different $MgCl_2$ and *Taq* polymerase concentrations. (A) Results of the second PCR products amplified at concentration of $MgCl_2$ 2.0 mM and *Taq* polymerase 1.0 U showing the presence of non-specific bands in all PCR sets. (B) Second PCR products amplified at a concentration of $MgCl_2$ 1.0 mM and *Taq* polymerase 0.5 U. Results showed that $MgCl_2$ and *Taq* polymerase concentrations have a significant effect on elimination of the non-specific bands, and all the expected bands were visualised clearly. M: 100-bp DNA ladder; Wt: wild-type; Mt: mutant-type.

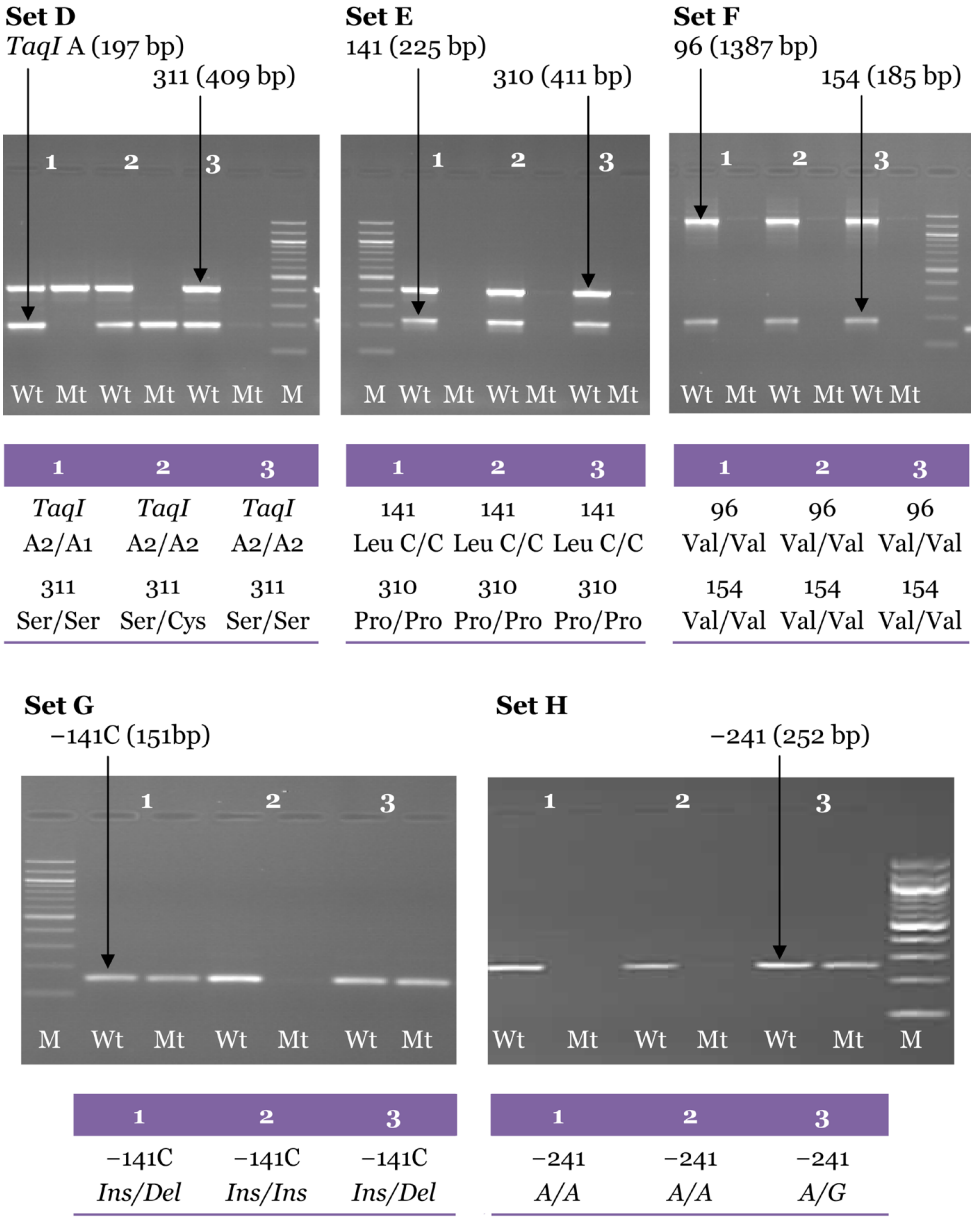


Figure 6: Electrophoresis pattern for the second PCR amplification from 3 genomic DNA samples carried out with the specific primers using the final optimised PCR for Set D (Ser311Cys and *TaqI* A), Set E (Pro310Ser, Leu141Leu), Set F (Val96Ala and Val154Ile), Set G (−141C *Ins/Del*) and Set H (*A*-241*G*). Each pair of lanes represents 1 sample. The genotypes for each sample are shown in the tables. M: 100-bp DNA ladder; Wt: wild-type; Mt: mutant-type.

from patients with schizophrenia (23). The mutation sites detected by this method were further confirmed with sequencing results. The sequencing analysis showed 100% homology to *DRD2*, and the results from the second PCR obtained from the samples also corresponded with the sequencing analysis. An example of our sequencing results is illustrated in Figure 7.

Discussion

Currently, the time-consuming and labour-intensive PCR–RFLP is one of the most commonly used methods for the determination of genetic polymorphisms of the DRD2 gene. Apart from requiring highly skilled personnel, if the polymorphism does not involve a restriction site

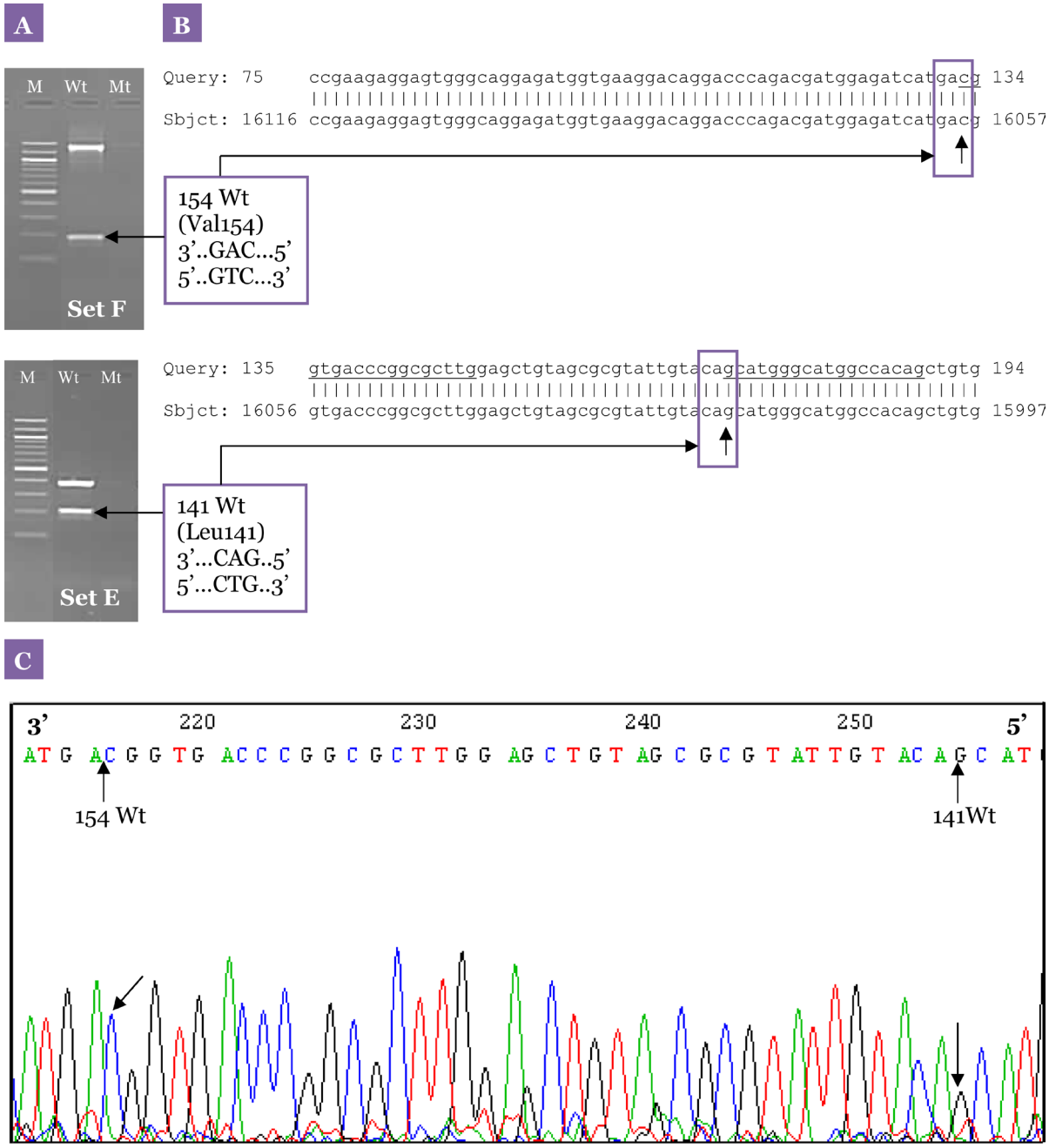


Figure 7: Specificity of the primer sets for the optimised nested allele-specific PCR for *DRD2* polymorphisms. (A) A representative agarose gel electrophoresis of the multiplex allele-specific PCR analysis of Set F and Set E showing no mutant-type alleles detected. M: 100-bp DNA ladder; Wt: wild-type; Mt: mutant-type. (B) Nucleotide sequence of the PCR product obtained from sequencing was aligned using BLAST. The result was then compared with gel electrophoresis results of the selected sample, which confirmed the variation sites at codon 141 and 154 of the human *DRD2* for Leu141Leu and Val154Ile polymorphisms, respectively. The locations of the allele-specific second PCR primers are underlined. The nucleotide sequence is numbered from the 3'-end of *DRD2* as indicated at both sides of each line. (C) Chromatogram of nucleotide sequences from the sample showing homozygous wild-type alleles for Leu141Leu and Val154Ile polymorphisms. The position of the Leu141Leu and Val154Ile polymorphisms is indicated by the mark symbols and the vertical arrows.

change, PCR–RFLP cannot be used. In contrast, allele-specific multiplex PCRs do not require restriction enzyme cleavage and are less time-consuming as well as more user-friendly.

Apart from its advantages over PCR–RFLP, multiplex PCR, as developed in this study, is a promising technique to overcome other shortcomings of single PCR reactions and thus can increase the diagnostic capacity of PCR. In a multiplex PCR, more than 1 target sequence is amplified by including more primer pairs in a PCR reaction (24). Multiplex PCR thus has the potential to save time and reduce tedious laboratory work without compromising test accuracy. To our knowledge, this is the first report of a method for the simultaneous detection of multiple *DRD2* polymorphisms using nested allele-specific multiplex PCR with the same conditions of thermal cycling.

Three main components that are important for the success of multiplex PCR are the cycling conditions, the PCR mixture, and the characteristics of the designed primers (25,26). Primers are the primary components of a PCR. The choice of specific primers will ensure the specificity of the PCR method because a non-exact match with the sample DNA may cause false amplification. We opted to use a nested multiplex PCR approach to amplify the *DRD2* regions of interest in the first PCR for use as a template in the second allele-specific PCR. Primers were designed to yield PCR products of different sizes so that identification was easier by gel electrophoresis. As a rule-of-thumb, it is advisable to design primers with a similar T_m of 55 to 58 °C or higher, of 18 to 30 bp or higher in length, and of GC content of 35% to 60% for easier optimisation (24). These designed primers were aligned using BLAST programme and were shown to be 100% specific for *DRD2*. The second PCR gel pictures (Figure 4) showed no significant primer dimers or large allelic noise bands, which indicated that the designed primer sets were PCR compatible. The specificity of the primers was further confirmed with sequencing analysis.

Alterations in the PCR mixture and the cycling conditions resulted in improvement in the sensitivity and specificity of the method developed. The recommended PCR cycles were between 25 and 35 (27). Thirty-five cycles of PCR were used for the first PCR to ensure sensitivity. However, increasing the number of cycles will also increase amplification of non-specific products and analysis time. The cycles chosen for the first PCR were sufficient to allow for the second PCR. Subsequently, 15 cycles of PCR were performed for detection of variants.

Annealing temperature is an important parameter for PCR optimisation. If the annealing temperature is too high, no annealing can occur, and an annealing temperature that is too low will result in an increase in non-specific annealing (27). *Taq* polymerase is another important factor to be optimised along with magnesium and dNTPs. One of the reasons for its importance is the expense, and another reason is the fact that fidelity (nucleotide mis-incorporation frequency) of *Taq* polymerase depends upon the concentration of free Mg^{2+} and dNTP. For most PCRs, the optimum amount of *Taq* polymerase will be between 0.5 to 2.5 U in a 50.0 µL reaction volume (27).

In this study, 3 separate sets of the first PCR mixture were designed to amplify a region from exon 3 to 4 (Set A), exon 7 and *TaqI* A (Set B), and the promoter region (Set C) instead of just 1 PCR mixture. The initial 4 primer pairs in 1 set resulted in problems with the second PCR. The problems included amplification of non-specific products, multiple product yield (first PCR product carried over), high molecular weight smears, primer dimmers, and failure to amplify.

Similarly, combining the designed primer pairs to yield just 3 reaction mixtures for use in a multiplex second PCR was not successful. The combinations of primer pairs attempted were Val96Ala, Ser311Cys, and *TaqI* A (Set 1); Pro310Ser, Leu141Leu, and Val154Ile (Set 2); and the promoter region polymorphisms, *A*-241*G*, and –141C *Ins/Del* (Set 3). The resulting multiplexes were not reproducible especially for Val96Ala, Val154Ile, and –141C *Ins/Del*. Val96Ala and Val154Ile were taken out from the sets and combined in a separate reaction mixture. Similarly, –141C *Ins/Del* was separated from *A*-241*G*, which resulted in 2 uniplex mixtures for the promoter region. Other primer pair combinations were tried but were also not reproducible. Splitting the multiplex reactions into 5 final sets made optimisation of the PCR easier, and the results obtained were more specific and reproducible.

The optimised methods were found to be robust when repeated by several individuals at the laboratory at the Faculty of Pharmacy, Universiti Teknologi MARA, to test for inter-individual robustness. The gel images were analysed by 2 independent, blinded reviewers to ensure unequivocal results. Samples that did not have clear bands or equivocal results were repeated. The PCR results were subsequently confirmed by direct sequencing.

Overall, the final allele-specific multiplex PCR method was the result of condition optimisation of factors such as primer selection,

quality of the DNA, magnesium amount, *Taq* polymerase amount, and annealing temperature. Our method was found to be specific in detecting 8 *DRD2* polymorphisms (Val96Ala, Leu141Leu, Val154Ile, Ser311Cys, Pro310Ser, *TaqI* A, *A*-241*G*, and –141C *Ins/Del*). The specificity of the method was further confirmed with sequencing analysis (Figure 7).

Recently, a new genotyping method has been described that uses microchip electrophoresis for the analysis of PCR products for sizing, mutations, or polymorphisms. For example, this method can be used in detecting the methylated *p16* gene in cancer patients (28), the analysis of the dopamine D4 receptor gene polymorphism (29) and the detection and identification of yeast strains (30). The microchip electrophoretic system is faster and more specific when compared with agarose gel electrophoresis (28). Barta et al. (29) confirmed the reliability of the new separation method by comparing it to the conventional slab gel and the ultra-thin-layer techniques in genotyping the 48 bp repeat polymorphism in the dopamine D4 receptor gene (*DRD4*). On the other hand, no differences in discriminating power or sensitivity were observed between the PCR-agarose gel electrophoresis method and the PCR-microchip electrophoresis method in identification of common and uncommon yeast strains (30). To the best of our knowledge, there has been no study in which the microchip electrophoresis analysis system was used in a clinical or research laboratory for determining the lengths of PCR products for simultaneous detection of *DRD2* polymorphisms. This new approach requires sophisticated techniques and expensive equipment that are not usually available in most research and diagnostic settings, hence the limited implementation of this method. The nested multiplex allele-specific PCR in this study offers a simple, inexpensive, and specific method that does not require additional equipment or additional complexity. The only step involved after PCR is the conventional agarose gel electrophoresis for interpretation of the PCR products. Furthermore, agarose gel electrophoresis is the technique of choice for many laboratories without the need for costly reagents and equipment.

Conclusion

We have developed a nested multiplex allele-specific PCR for the simultaneous detection of *DRD2* polymorphisms and sufficient information about the assay has been provided. Our ability to simultaneously amplify more than 1 locus in the same reaction in a multiplex PCR is desirable for population studies.

Acknowledgements

This study was supported by the IRPA PR (RM8) Grant No. 06-02-05-1015PR002, Ministry of Science, Technology, and Environment Malaysia under the project "Value Added Therapeutic Drug Monitoring: Application of Pharmacogenomics in the Management of Mental Illness". We thank all the members in Pharmacogenetics Research Group, Institute for Research in Molecular Medicine, Universiti Sains Malaysia, and in Faculty of Pharmacy, Universiti Teknologi MARA, for their invaluable contributions to the project.

Authors' Contributions

Conception and design, provision of study materials, analysis and interpretation of the data, critical revision and final approval of the article: ZZ, MRS, MKZJ, NM, RI
Obtaining of funding: MRS, RI
Collection and assembly of data, drafting of the article: ZZ
Administrative, technical, or logistic support: RI

Correspondence

Ms Zalina Zahari
BSc Pharmacy (Hons) (Strathclyde),
MSc Pharmacogenetics (USM)
Department of Pharmacy
Hospital Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan, Malaysia
Tel: +609-767 3416
Fax: +609-767 3377
Email: zzalina@kb.usm.my

References

1. Neville MJ, Johnstone EC, Walton RT. Identification and characterization of *ANKK1*: A novel kinase gene closely linked to *DRD2* on chromosome band 11q23.1. Hum Mutat. 2004;**23(6)**:540–545

2. Jackson DM, Westlind-Danielsson A. Dopamine receptors: Molecular biology, biochemistry and behavioural aspects. *Pharmacol Ther*. 1994;**64(2)**:291–370.

3. Grandy DK, Marchionni MA, Makam H, Stofko RE, Alfano M, Frothingham L, et al. Cloning of the cDNA and gene for a human D2 dopamine receptor. *Proc Natl Acad Sci U S A*. 1989;**86(24)**:9762–9766.

4. Eubanks JH, Djabali M, Selleri L, Grandy DK, Civelli O, McElligott DL, et al. Structure and linkage of the D2 dopamine receptor and neural cell adhesion molecule genes on human chromosome 11q23. *Genomics*. 1992;**14(4)**:1010–1018.

5. Itokawa M, Arinami T, Futamura N, Hamaguchi H, Toru M. A structural polymorphism of human dopamine D2 receptor, D2 (Ser311→Cys). *Biochem Biophys Res Commun*. 1993;**196(3)**:1369–1375.

6. Gejman PV, Ram A, Gelernter J, Friedman E, Cao Q, Pickar D, et al. No structural mutation in the dopamine D2 receptor gene in alcoholism or schizophrenia. Analysis using denaturing gradient gel electrophoresis. *JAMA*. 1994;**271(3)**:204–208.

7. Cravchik A, Sibley DR, Gejman PV. Functional analysis of the human D2 dopamine receptor missense variants. *J Biol Chem*. 1996;**271(42)**:26013–26017.

8. Arinami T, Gao M, Hamaguchi H, Toru M. A functional polymorphism in the promoter region of the dopamine D2 receptor gene is associated with schizophrenia. *Hum Mol Genet*. 1997;**6(4)**:577–582.

9. Thompson J, Thomas N, Singleton A, Piggott M, Lloyd S, Perry EK, et al. D2 dopamine receptor gene *(DRD2) Taq1 A* polymorphism: Reduced dopamine D2 receptor binding in the human striatum associated with the A1 allele. *Pharmacogenetics*. 1997;**7(6)**: 479–484.

10. Jonsson EG, Nothen MM, Grunhage F, Farde L, Nakashima Y, Propping P, et al. Polymorphisms in the dopamine D2 receptor gene and their relationships to striatal dopamine receptor density of healthy volunteers. *Mol Psychiatry*. 1999;**4(3)**:290–296.

11. Blum K, Noble EP, Sheridan PJ, Montgomery A, Ritchie T, Jagadeeswaran P, et al. Allelic association of human dopamine D2 receptor gene in alcoholism. *JAMA*. 1990;**263(15)**:2055–2060.

12. Li MD, Ma JZ, Beuten J. Progress in searching for susceptibility loci and genes for smoking-related behaviour. *Clin Genet*. 2004;**66(5)**:382–392.

13. Wang J, Liu ZL, Chen B. Association study of dopamine D2, D3 receptor gene polymorphisms with motor fluctuations in PD. *Neurology*. 2001;**56(12)**: 1757–1759.

14. Schindler KM, Pato MT, Dourado A, Macedo A, Azevedo MH, Kennedy JL, et al. Association and linkage disequilibrium between a functional polymorphism of the dopamine-2 receptor gene and schizophrenia in a genetically homogeneous Portuguese population. *Mol Psychiatry*. 2002;**7(9)**:1002–1005.

15. Lencz T, Robinson DG, Xu K, Ekholm J, Sevy S, Gunduz-Bruce H, et al. DRD2 promoter region variation as a predictor of sustained response to antipsychotic medication in first-episode schizophrenia patients. *Am J Psychiatry*. 2006;**163(3)**:529–531.

16. Hauge XY, Grandy DK, Eubanks JH, Evans GA, Civelli O, Litt M. Detection and characterization of additional DNA polymorphisms in the dopamine D2 receptor gene. *Genomics*. 1991;**10(3)**:527–530.

17. Grandy DK, Zhang Y, Civelli O. PCR detection of the *Taq*A RFLP at the *DRD2* locus. *Hum Mol Genet*. 1993;**2(12)**:2197.

18. Arinami T, Itokawa M, Enguchi H, Tagaya H, Yano S, Shimizu H, et al. Association of dopamine D2 receptor molecular variant with schizophrenia. *Lancet*. 1994;**343(8899)**:703–704.

19. Hori H, Ohmori O, Shinkai T, Kojima H, Nakamura J. Association analysis between two functional dopamine D2 receptor gene polymorphisms and schizophrenia. *Am J Med Genet*. 2001;**105(2)**: 176–178.

20. Grandy DK, Litt M, Allen L, Bunzow JR, Marchionni M, Makam H et al. The human dopamine D2 receptor gene is located on chromosome 11 at q22-q23 and identifies a *Taq*I RFLP. *Am J Hum Genet*. 1989;**45(5)**:778–785.

21. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;**16(3)**:1215.

22. Murakami N, Sirugo G, Seaman MI, Bray-Ward P, Pakstis AJ, Kidd KK. Characterization of the 5'-flanking region of the DRD2 gene including a (GAAA)n STRP. *Am J Hum Genet*. 1998;**63(Suppl)**:A302(1748).

23. Zahari Z, Teh LK, Ismail R, Razali SM. Influence of *DRD2* polymorphisms on the clinical outcomes of patients with schizophrenia. *Psychiatr Genet*. 2011;**21(4)**:183–189.

24. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques*. 1997;**23(3)**: 504–511.

25. Zainuddin Z, Teh LK, Suhaimi AW, Salleh MZ, Ismail R. A simple method for the detection of *CYP2C9* polymorphisms: Nested allele-specific multiplex polymerase chain reaction. *Clin Chim Acta*. 2003;**336(1–2)**:97–102.

26. Richards CS, Bradley LA, Amos J, Allitto B, Grody WW, Maddalena A, et al. Standards and guidelines for CFTR mutation testing. *Genet Med*. 2002;**4(5)**: 379–391.

27. Rolf A, Schuller I, Finckh U, Weber-Rolfs I. *PCR: Diagnostics and research*. Berlin (DE): Springer-Verlag Telos; 1992.

28. Zhou XM, Shao SJ, Xu GD, Zhong RT, Liu DY, Tang JW, et al. Highly sensitive determination of the methylated *p16* gene in cancer patients by microchip electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;**816(1–2)**:145–151.

29. Barta C, Ronai Z, Nemoda Z, Szekely A, Kovacs E, Sasvari-Szekely M, et al. Analysis of dopamine D4 receptor gene polymorphism using microchip electrophoresis. *J Chromatogr A*. 2001;**924 (1–2)**:285–290.

30. Fujita SI, Senda Y, Nakaguchi S, Hashimoto T. Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains. *J Clin Microbiol*. 2001;**39(10)**: 3617–3622.

Original Article

Neurodevelopmental Outcome of Newborns with Persistent Pulmonary Hypertension

Jaafar Rohana[1], Nem Yun Boo[2], Viji Chandran[1], Rajini Sarvananthan[1]

Submitted: 6 Dec 2010
Accepted: 3 Jan 2011

[1] *Department of Paediatrics, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia*

[2] *Department of Clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Jalan Sungai Long, 43000 Kajang, Selangor, Malaysia*

Abstract

Background: **Developmental disabilities have been reported in infants with persistent pulmonary hypertension of the newborn (PPHN) treated with inhaled nitric oxide (INO) or intravenous magnesium sulphate ($MgSO_4$) and/or extracorporeal membrane oxygenation. This paper reports the rate of developmental disabilities at 2 years of age in a cohort of survivors of PPHN treated with INO, $MgSO_4$, or both during the neonatal period.**

Methods: **Sixteen survivors of PPHN were prospectively followed up. These infants were treated with intravenous $MgSO_4$ and/or INO during the neonatal period. Neurodevelopmental assessment was carried out at 2 years of age using the Bayley Scales of Infant Development 2nd Edition by a developmental psychologist. Eleven (68.8%) infants completed the 2-year follow-up.**

Results: **The median mental developmental index (MDI) and physical developmental index scores were 85 (interquartile range, IQR = 27) and 87 (IQR = 33), respectively. Two infants (18.2%) had developmental disability (MDI scores <70).**

Conclusion: **Survivors of PPHN are at risk of developmental disabilities. Early intervention programme and long-term follow-up should be integrated in the management of these infants.**

Keywords: *developmental disabilities, magnesium sulphate, neurology, nitric oxide, persistent pulmonary hypertension of newborn*

Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome that is associated with a variety of neonatal cardiopulmonary diseases, including meconium aspiration syndrome (MAS), hyaline membrane disease, pneumonia, and congenital diaphragmatic hernia. Significant hypoxaemia with right-to-left shunting across a patent foramen ovale, ductus arteriosus, or both, are clinical hallmarks of PPHN. The mortality rate was high (11%–48%), and PPHN is the most common indication for infants needing extracorporeal membrane oxygenation, ECMO (1).

Recently, new therapeutic modalities, such as inhaled nitric oxide (INO), have been used in the treatment of PPHN (2). INO acts as a pulmonary vasodilator by activating guanylate cyclase, which leads to an increase in the production of cyclic guanosine monophosphate that causes vascular smooth muscle relaxation. However, INO therapy is expensive and only widely available in developed countries. Intravenous magnesium sulphate ($MgSO_4$) has been used as a vasodilator in the treatment of PPHN in some developing and developed countries (3–5). The disadvantage of $MgSO_4$ treatment is that it causes systemic vasodilation, and hypotension is a common side effect.

Infants with PPHN become critically ill during the neonatal period and are at risk for adverse neurodevelopmental outcomes. Severe neurodevelopmental disability was reported to occur in 11.8% by 1 year of age and 12.1% by 2 years of age in a group of infants who was treated for PPHN with INO during the neonatal period (6). Lipkin et al. (7) reported the outcome at 1 year of age in 133 infants with moderately severe PPHN who were treated with INO. Major neurologic abnormalities, cognitive delays, and hearing loss were present in 13%, 30%, and 19% of infants, respectively. Tolsa et al. (4) showed that 11 infants with PPHN who were treated with intravenous $MgSO_4$ had normal neurodevelopment at 6 and 12 months of age. However, in another study

(5), major neurodevelopment impairments in preschool-age children were reported to occur in 11.5% of children who were treated with $MgSO_4$ for PPHN.

This study examined the neurodevelopmental outcome at 2 years of age for a group of PPHN survivors who were treated with intravenous $MgSO_4$, INO, or both during the neonatal period.

Subjects and Methods

Thirty-eight term and near-term infants (more than 34 gestational weeks) with PPHN were admitted to the Neonatal Intensive Care Unit, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, over a 3-year period between April 2000 and April 2003. The unit is a level III neonatal intensive care unit that offers a wide range of services, except ECMO. Twenty-five (65.8%) of the infants were involved in a randomised controlled trial (RCT) comparing the effectiveness of intravenous $MgSO_4$ and INO in term infants with PPHN (8).

The PPHN diagnosis was based on the echocardiographical evidence of right-to-left shunting of blood across the ductus arteriosus and/or the foramen ovale in newborns with severe hypoxaemia (oxygenation index, OI, of more than 25). All infants were on a high frequency oscillatory ventilator (Model 3100A; Sensor Medics, Yorba Linda, CA) support.

The infants in the RCT were managed according to the following protocol. Infants in the intravenous $MgSO_4$ group were administered a loading dose of $MgSO_4$ at 200 mg/kg infused over half an hour, followed by a continuous infusion of $MgSO_4$ at 50–150 mg/kg/hour to achieve a serum magnesium level of 5.0–7.0 mmol/L. Infants in the INO group were administered an initial dose of 20 ppm via the INOvent delivery system (Datex-Ohmeda, Madison, WI, USA). The vasodilator treatment was switched to the other vasodilator when the infant developed acute deterioration in the pulse oximetry or in the OI during the first 4 hours of treatment, or if the right-to-left shunt persisted beyond 12 hours of life. The first vasodilator was removed over 12–24 hours. The vasodilator that the infant responded to was removed once the PPHN resolved based on the echocardiographical findings, and the infants tolerated a lower high-frequency oscillatory ventilation setting with a mean airway pressure of 8 cm H_2O or less and a fraction of inspired O_2 of 0.5 or less. The infusion of $MgSO_4$ was gradually decreased over the next 24 hours. The INO was reduced by 5 ppm every 12 hours until it reached 5 ppm, and then it was reduced by 1 ppm every 4 hours. Serial chest radiographs were used to ensure optimal lung inflation to the $8^{1/2}$ or 9th rib. The level of pCO_2 was kept between 35 and 50 mmHg. Infusions of dopamine and/or dobutamine were used to maintain normal blood pressure (mean arterial pressure at the 50th percentile for gestational age). Metabolic acidosis was treated with sodium bicarbonate.

Infants whose parents did not provide consent for the RCT were managed in the same manner as the infants involved in the RCT except for the choice of vasodilator therapy. They were started on INO, and $MgSO_4$ infusion was used as the first vasodilator when contraindications to INO (e.g., coagulopathy and bleeding diathesis) were present. The infants who did not respond to INO were switched to the $MgSO_4$ infusion. The same weaning strategy was used for these infants.

Follow-ups for the survivors occurred at our clinic at 3, 6, 12, 18, and 24 months of age. During these visits, parents were interviewed regarding their children's feeding, intercurrent illnesses, re-hospitalisations, and developmental milestones. The growth parameters were measured and physical and neurological examinations (Amiel-Tison et al., 9) were performed by the neonatologists. Hearing tests using brainstem auditory evoked potentials were performed within 6 months of being discharged from the hospital. The hearing test was repeated at 1 year of age if the first test was normal, but earlier if it showed any abnormality. At 24 months of age, the children were assessed using the Bayley Scales (2nd edition), by a clinical psychologist who was not involved in the care of these children during the neonatal period. For an infant who was born at or before 36 weeks of gestation, the assessment was performed at 24 months for the corrected age.

Abnormal growth was defined as body weight and length below the 10th percentile on the National Centre for Health Statistics growth chart. An adverse neurodevelopmental outcome was defined as a score of less than 70 on either portion of the Bayley Scales, an abnormal finding on the neurological examination, or both.

The data were analysed using the SPSS version 12.0.1 (SPSS Inc., Chicago, IL). The Mann–Whitney U test was used to compare the findings between infants receiving different vasodilator therapies. A P value of less than 0.05 was considered significant.

Results

Overall, 16 (42.1%) of the 38 infants survived until discharge. However, only 11 (68.75%) survivors completed the 2-year follow–up; 7 of these infants were involved in the RCT. The other 5 survivors defaulted on the follow-up and could not be located. The demographic characteristics and clinical data of the infants are shown in Table 1. The majority of the infants were males (n = 8, 72.7%). Their mean gestation and median birth weight were 39 weeks (SD 1.89) and 3050 g (IQR 2720, 3670), respectively, and 9 (81.8%) had MAS.

Seven (63.6%) of the infants received both INO and $MgSO_4$, and 4 infants received INO alone (Table 2). The median mental developmental index (MDI) and psychomotor developmental index (PDI) scores of the survivors were 85 (IQR 27) and 87 (IQR 33), respectively. Two infants scored less than 70 on the Bayley's testing for MDI (Table 2). One of them (infant no. 4) had moderate PPHN (maximum OI of 39) and was ventilated for 21 days. The other (infant no. 9) had grade 2 hypoxic ischaemic encephalopathy according to modified Sarnat staging (10); this infant also had severe PPHN (maximum OI of 160), and the OI decreased to less than 25 only after 67 hours. Both infants received INO and $MgSO_4$ therapy.

Even though the number of infants who completed the follow-up was small, we attempted to compare the outcome in infants who received INO alone with those who received the combined INO and $MgSO_4$ therapy. In comparison with the infants who received INO alone, those who received the combined therapy had higher OI levels, with a median (IQR) of 53.2 (20.6), and a longer duration of OI of more than 25, with a median (IQR) of 44 hours (51.2), compared with those who received the INO alone, with a median (IQR) of 38.9 (27.5) for OI level (P = 0.4) and 14.5 hours (13.0) for duration of OI of more than 25 (P = 0.4). The infants who received the combined therapy had higher MDI scores, with a median (IQR) of 92 (45), and higher PDI scores, with a median (IQR) of 92 (48), compared with those receiving INO alone, with a median (IQR) of 84.5 (17.5) for MDI score and 85.5 (12) for PDI score; however, these differences were not statistically significant (P = 0.9 for MDI and P = 0.5 for PDI).

None of the 11 survivors had feeding difficulties, eye/hearing problems, or an abnormal neurological examination. One infant had failure to thrive, whereas 4 (36.4%) had hyper-reactive airway problems.

Table 1: Demographic and clinical data from survivors of persistent pulmonary hypertension of the newborn

Case no.	Sex	Gestation (weeks)	Birth weight (g)	5-minute Apgar score	Respiratory diagnosis
1	F	39	37003	10	MAS
2	M	41	3300	9	MAS
3	F	39	2850	5	MAS
4	M	42	3670	9	MAS
5	M	38	3050	8	MAS
6	F	38	2800	10	Pneumonia
7	M	39	3360	8	MAS
8	M	35	2080	9	RDS
9	M	39	3740	6	MAS
10	M	38	2720	9	MAS
11	M	41	2605	9	MAS

Abbreviations: M = male, F = female, MAS = meconium aspiration syndrome, RDS = respiratory distress syndrome

Table 2: Vasodilator therapy, clinical parameters, and mental and physical developmental index scores of the survivors of persistent pulmonary hypertension of the newborn

Case no.	Vasodilator	Highest OI	Duration of OI > 25 (hours)	Duration of ventilation (days)	MDI score	PDI score
1	INO + $MgSO_4$	89.8	78.0	8.0	114	92
2	INO	28.0	13.0	4.0	81	73
3	INO + $MgSO_4$	32.0	n/a	11.0	92	125
4 *	INO + $MgSO_4$	39.0	32.0	21.5	58	77
5	INO	37.7	14.5.0	16.0	84	88
6	INO + $MgSO_4$	n/a	22.0	10.0	103	121
7	INO + $MgSO_4$	59.0	56.0	21.0	100	106
8	INO	63.8	n/a	12.0	85	87
9 *	INO + $MgSO_4$	160.0	67.0	10.0	50	54
10	INO + $MgSO_4$	47.4	8.5	6.0	76	73
11	INO	40.0	47.0	5.0	104	84

* Infants with MDI score of less than 70.
Abbreviation: INO = inhaled nitric oxide, $MgSO_4$ = magnesium sulphate, OI = oxygenation index, MDI = mental developmental index, PDI = psychomotor developmental index, n/a = not available

Discussion

The rate of an adverse neurodevelopmental outcome at 2 years of age in our survivors was 18.2%. However, comparing our findings with previously reported neurodevelopmental outcomes is difficult because of the differences in the criteria for the treatment and management strategies and outcome measures (3–6,11–13) Many studies examined infants who were treated with either $MgSO_4$ or INO. The majority (63.6%) of our patients received both vasodilators. The infants in our study were very ill, which was demonstrated by a high median OI of 53.2. The majority of these infants met the eligibility criteria for an ECMO referral (OI of 40 or more). Ichiba et al. (11) reported a 6.7% adverse outcome rate at 3 years of age among the 15 survivors with moderately severe PPHN (mean OI 27.2, SD 15.2). Furthermore, they reported that a normal neurodevelopmental outcome was significantly higher in the infants who responded early to the treatment (OI of less than 10 within 1 hour). The majority of our patients had a high OI for prolonged periods. Because of the small sample size, it was not possible to determine whether a combination of INO and $MgSO_4$ therapy was associated with a better neurodevelopmental outcome, although the trend suggested that the combination therapy was associated with a higher MDI and PDI in infants with a more severe PPHN.

These infants should undergo a longer term follow-up evaluation because they are still at risk of neurodevelopmental impairment and behavioural problems. Galli et al. (4) found a major impairment in 6% at 18 months and 11.4% at 5 years old in a group of 33 infants with PPHN who were treated with $MgSO_4$. Behavioural problems were reported to be higher among 2- to 4-year-old PPHN survivors than the rate of 14% among reference populations (12). Berti et al. (13) reported that 26% and 22% of the PPHN survivors who were assessed at a mean age of 41 months had behavioural problems and language disturbances, respectively.

Conclusion

Our study indicated that the survivors of PPHN have a high risk of adverse neurodevelopmental outcomes. Early intervention program and long term follow-up should be integrated in the management of these infants.

Authors' Contributions

Conception and design, obtaining of funding, critical revision of the article: NYB
Provision of patients: VC, RS
Collection and assembly of the data: NYB, VC, RS, JR
Analysis and interpretation of the data: JR, NYB
Drafting of the article: JR

Correspondence

Dr Rohana Jaafar
MMed Paeds (UKM)
Department of Paediatrics
Universiti Kebangsaan Malaysia Medical Centre
Jalan Yaacob Latif, Bandar Tun Razak
56000 Cheras
Kuala Lumpur, Malaysia
Tel: +603-9145 7822
Fax: +603-9145 6637
Email: drohana@ppukm.ukm.my

References

1. Clark RH. High-frequency ventilation. *J Pediatr*. 1994;**124 (5 Pt 1)**:661–670.

2. Kinsella JP, Truog WE, Walsh WE, Goldberg RN, Bancalari E, Mayock DE, et al. Randomized, multicenter trial of inhaled nitric oxide and high-frequency oscillatory ventilation in severe, persistent pulmonary hypertension of the newborn. *J Pediatr*. 1997;**131(1)**:55–62.

3. Abu-Osba YK, Galai O, Manasra K, Rejjal A. Treatment of severe persistent pulmonary hypertension of the newborn with magnesium sulphate. *Arch Dis Child*. 1992;**67(1 Spec No)**:31–35.

4. Tolsa JF, Cotting J, Sekarsi N, Payot M, Micheli JL, Calame A. Magnesium sulphate as an alternative and safe treatment for severe persistent pulmonary hypertension of the newborn. *Arch Dis Child Fetal Neonatal Ed*. 1995;**72(3)**:F184–187.

5. Galli S, Bickle Graz M, Forcada-Guex M, Muehlethaler V, Jaunin L, Tolsa JF. Neuro-developmental follow-up of neonates treated with magnesium sulphate for persistent pulmonary hypertension. *J Neonat Perinat Med*. 2008;**1(2)**:83–91.

6. Rosenberg AA, Kennaugh JM, Moreland SG, Fashaw LM, Hale KA, Torielli FM, et al. Longitudinal follow-up of a cohort of newborn infants treated with inhaled nitric oxide for persistent pulmonary hypertension. *J Pediatr*. 1997;**131(1 Pt 1)**:70–75.

7. Lipkin PH, Davidson D, Spivak L, Straube R, Rhines J, Chang CT. Neurodevelopmental and medical outcomes of persistent pulmonary hypertension in term newborns treated with inhaled nitric oxide. *J Pediatr*. 2002;**140(3)**:306–310.

8. Boo N Y, Rohana J, Yong SC, Bilkis AZ, Yong-Junina F. Inhaled nitric oxide and intravenous magnesium sulphate for the treatment of persistent pulmonary hypertension of the newborn. *Singapore Med J*. 2010;**51(2)**:144–150.

9. Amiel-Tison C, Gosselin J. *Neurological development from birth to six years: Guide to examination and evaluation*. Baltimore (MD): The John Hopkins University Press; 2001.

10. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch Neurol*. 1976;**33(10)**:698–705.

11. Ichiba H, Matsunami S, Itoh F, Ueda T, Ohsasa Y, Yamano T. Three-year follow up of term and near term infants treated with inhaled nitric oxide. *Pediatr Int*. 2003;**45(3)**:290–293.

12. Ellington M Jr, O'Reilly D, Allred EN, McCormick MC, Wessel DL, Kourembanas S. Child health status, neurodevelopmental outcome, and parental satisfaction in a randomized, controlled trial of nitric oxide for persistent pulmonary hypertension of the newborn. *Pediatrics*. 2001;**107(6)**:1351–1356.

13. Berti A, Janes A, Furlan R, Macagno F. High prevalence of minor neurologic deficits in a long-term neurodevelopmental follow-up of children with severe persistent pulmonary hypertension of the newborn: A cohort study. *Ital J Pediatr*. 2010;**36**:45–51.

Original Article

A Preliminary Study on the Reliability of the Malay Version of PedsQL™ Family Impact Module among Caregivers of Children with Disabilities in Kelantan, Malaysia

Azriani Ab Rahman[1], Norsarwany Mohamad[2], Musa Kamarul Imran[1], Wan Pauzi Wan Ibrahim[2], Azizah Othman[2], Aniza Abd Aziz[3], Sakinah Harith[4], Mohd Ismail Ibrahim[1], Nor Hashimah Ariffin[1], Hans Van Rostenberghe[2]

Submitted: 7 Feb 2011
Accepted: 29 May 2011

[1] *Department of Community Medicine, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[2] *Department of Pediatrics, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[3] *Biostatistics and Research Methodology Unit, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[4] *School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

Abstract

Background: **No previous study has assessed the impact of childhood disability on parents and family in the context of Malaysia, and no instrument to measure this impact has previously been available. The objective of this cross-sectional study was to determine the reliability of a Malay version of the PedsQL™ Family Impact Module that measures the impact of children with disabilities (CWD) on their parents and family in a Malaysian context.**

Methods: **The study was conducted in 2009. The questionnaire was translated forward and backward before it was administered to 44 caregivers of CWD to determine the internal consistency reliability. The test for Cronbach's alpha was performed.**

Results: **The internal consistency reliability was good. The Cronbach's alpha for all domains was above 0.7, ranging from 0.73 to 0.895.**

Conclusion: **The Malay version of the PedsQL™ Family Impact Module showed evidence of good internal consistency reliability. However, future studies with a larger sample size are necessary before the module can be recommended as a tool to measure the impact of disability on Malay-speaking Malaysian families.**

Keywords: *caregivers, disabled children, public health, psychometrics, quality of life, questionnaires, reliability and validity*

Introduction

With the advancement of treatment modalities, more children survive severe acute illnesses but, often, not without sequelae causing some form of chronic morbidity or disability. The care of chronically ill and disabled children is complex. Follow-up by a multidisciplinary team is often desirable, but in many places in Malaysia, such services are not available. The need for multiple visits to medical care facilities and the problems faced by these children in carrying out their daily activities may have a significant impact on the family.

Several studies evaluating the impact of children's chronic morbidity and disability on the family have shown the presence of negative outcomes. Problems include social stigma, altered family dynamics, emotional disturbance (1), and psychosocial impact (2). However, the patient's characteristics and family structure and the disability services available in Malaysia may differ from the countries where these studies were conducted; as such, the impact on the families may differ in nature and magnitude.

For English-speaking populations, there are various instruments available to measure the impact of a child's disability on parents

and the family. These instruments include the Beck Depression Inventory, State-Trait Anxiety Inventory, the Nottingham Health Profile Part 1 (3), and the PedsQL™ Family Impact Module. The PedsQL™ Family Impact Module was designed to measure the impact of paediatric chronic health conditions on parents and the family. It measures parents' self-reported physical, emotional, social, and cognitive functions, communication, worry, parent-reported family daily activities, and family relationships (4).

To the best of our knowledge, there is no validated instrument for the Malaysian population that measures the impact of paediatric chronic illness or disability on parent and family functioning. This study was performed to determine the reliability of the Malay version of the PedsQL™ Family Impact Module, which measures the impact of children with disabilities (CWD) on their parents and family in a Malay-speaking population.

Materials and Methods

PedsQL™ Family Impact Module and setting

This study was conducted in Kelantan, which is situated in the northeast of Peninsular Malaysia. Consent from the original authors of the PedsQL™ Family Impact Module was sought to use this module and to create and validate a Malay version of it. This module measures parents' self-reported physical functioning (6 items), emotional functioning (5 items), social functioning (4 items), cognitive functioning (4 items), communication (3 items), and worry (5 items). It also measures parent-reported family daily activities (3 items) and family relationships (5 items). A 5-point response scale is utilised (0 = never a problem; 4 = always a problem). Items are reverse-scored and linearly transformed to a 0–100 scale (0 = 100, 1 = 75, 2 = 50, 3 = 25, 4 = 0) so that higher scores indicate better functioning (less negative impact). The original version of the PedsQL™ Family Impact Module was validated in San Diego among 23 families of medically fragile children with complex chronic health conditions who either resided in a long-term care convalescent hospital or resided at home with their families. The internal consistency was very good, as the Cronbach's alpha ranged from 0.82 to 0.97 (4).

Procedure

This study was approved by the Research and Ethics Committee, Universiti Sains Malaysia. The process of validation during the current study involved translation of the questionnaire and a cross-sectional field study among the caregivers of CWD. The original PedsQL™ Family Impact Module was translated from English to the Malay language independently by a medical doctor and a linguist who were fluent in both languages. The translations were revised and reconciled by the authors to produce a forward-translated version of the questionnaire. This version was back-translated into English by another medical doctor and linguist. The authors then compared the forward-translated and backward-translated versions and reconciled the questionnaire accordingly. The back-translated version was compared with the original PedsQL™ Family Impact Module to ensure its similarity. The authors agreed on the final version, and the face validity was determined to be acceptable.

The cross-sectional field study was conducted in September 2009 among caregivers of CWD and adolescents, aged 2 to 18 years old, who were registered with the Kelantan Foundation for the Disabled. Only participants who were able to understand the Malay language were included. The sample size was calculated using the ssalpha command based on Stata software (5). For a domain with the least number of items (3), with an expected Cronbach's alpha of 0.7 and a lower bound of Cronbach's alpha at 0.60 with 95% confidence, the required sample size was 95.

During the field study, the study rationale was explained to the caregivers, and informed consent was obtained before the caregivers answered the self-administered questionnaires.

Statistical analysis

Data entry and statistical analysis were conducted using SPSS version 12.1 (SPSS Inc., Chicago, IL). The demographic profile of the respondents was described using mean, SD, frequency, and percentage. We examined the mean and SD of each item in each domain to assess the item analysis. To determine the internal consistency reliability of the domains, the analysis for Cronbach's alpha was performed, and the item-total correlation was assessed. Cronbach's alpha above 0.7 was deemed to show acceptable internal consistency reliability (6–8).

Results

Profile of caregivers and children

A total of 44 caregivers were involved in this study; their demographic characteristics are listed in Table 1. All participants were Malay, and the majority of them were female (75.0%) and

attended formal education until secondary school (58.1%). Many of the participants were the parents of CWD (65.1%). The participants had a mean age of 48 years old (SD 10.6), and their median total monthly household income was RM900. The mean age of their CWD was 14 years old (SD 5.4). The children's types of disability included vision problems (n = 2), hearing impairment (n = 5), cerebral palsy and other physical disabilities (n = 8), learning problems including dyslexia (n = 13), and mental retardation including autism and Down syndrome (n = 17).

Means and standard deviations

Table 2 shows that the highest mean score was for the family relationship domain (82.6, SD 23.74), while the lowest mean score was for the physical domain (65.1, SD 22.88). The mean score of items ranged from 23.3 (SD 31.18) to 88.4 (SD 25.21). Among the items analysed, the highest mean score was for the item assessing the lack of communication among family members (family relationship domain). The lowest mean score was for the item assessing parents' worries about the future of their children (worry domain).

Internal consistency reliability

The internal consistency reliability based on the Cronbach's alpha of all domains was above 0.7 (ranging from 0.730 to 0.895). The highest Cronbach's alpha was for the family relationship domain, while the lowest was the communication domain. In general, the corrected item-total correlation for all domains was acceptable, at 0.4 and above, with the exception of the item "do not understand my family situation" (communication domain, item-total correlation 0.383). The corrected item-total correlation for the family relationship domain gave the highest Cronbach's alpha, ranging from 0.623 to 0.854. The detailed analyses are shown in Table 2.

Table 1: Demographic characteristics of caregivers and children

Characteristic	*n*	(%)
Sex of caregivers		
Male	11	(25.0)
Female	33	(75.0)
Age of caregivers	48	(10.6) [a]
Monthly household income	RM900	(1200) [b]
Marital status [c]		
Married	37	(88.1)
Divorced	5	(11.9)
Caregiver's level of education [d]		
No formal education	3	(7.0)
Primary school	14	(32.6)
Secondary school	25	(58.1)
Collage/university	1	(2.3)
Number of children	6	(2) [a]
Number of children with disabilities	1	(0.5) [a]
Age of children with disabilities	14	(5.4) [a]
Sex of children with disabilities		
Male	24	(54.5)
Female	20	(45.4)

[a] Results are expressed in mean (SD).
[b] Result is expressed in median (IQR), skewed to the right. The total number of subjects is 44.
[c] Marital status unknown for 2 subjects.
[d] Level of education is unknown for 1 subject.

Table 2: Item analysis and internal consistency reliability

Domains	Mean (SD)	I-T co	Cb's A
Physical functioning	65.1 (22.88)		0.845
Tired during the day	58.1 (31.21)	0.683	
Tired when waking up in the morning	59.3 (29.90)	0.646	
Too tired to do favourite things	66.3 (32.22)	0.591	
Headaches	61.6 (29.55)	0.680	
Physically weak	65.1 (32.80)	0.621	
Sick to the stomach	77.3 (27.72)	0.530	
Emotional functioning	71.9 (24.30)		0.850
Anxious	72.7 (33.55)	0.673	
Sad	65.1 (28.94)	0.679	
Angry	62.8 (26.9)	0.517	
Frustrated	78.5 (30.20)	0.783	
Helpless or hopeless	80.2 (33.91)	0.661	
Social functioning	76.6 (25.66)		0.822
Isolated from others	86.0 (25.18)	0.642	
Trouble getting support from others	74.4 (33.85)	0.633	
Difficult to find time for social activities	70.9 (34.50)	0.723	
Inadequate energy for social activities	75.0 (33.20)	0.613	
Cognitive functioning	69.3 (23.89)		0.823
Difficulty paying attention	68.0 (26.35)	0.405	
Difficulty remembering things people say	68.0 (28.00)	0.704	
Difficulty remembering things just heard	62.8 (34.23)	0.797	
Difficulty thinking quickly	78.5 (29.20)	0.715	
Communication	74.6 (23.14)		0.730
Others do not understand my family's situation	66.3 (29.32)	0.383	
Difficult to talk about the child's health	73.8 (29.40)	0.640	
Difficult to tell doctors and nurses their feeling	83.7 (27.21)	0.662	
Worry	63.4 (23.09)		0.788
The child's medical treatment is working or not	74.4 (30.61)	0.504	
Side effects of the medications/medical treatment	82.0 (26.90)	0.565	
Reaction of others to the child's condition	59.3 (34.10)	0.658	
The child's illness is affecting other family members	77.9 (34.60)	0.644	
The child's future	23.3 (31.18)	0.473	
Daily activities	69.3 (27.36)		0.771
Family activities take more time and effort	68.6 (38.22)	0.667	
Difficulty finding time to finish household tasks	74.4 (30.61)	0.546	
Feeling too tired to finish household tasks	66.9 (29.74)	0.629	
Family relationships	82.6 (23.74)		0.895
Lack of communication among family members	88.4 (25.21)	0.682	
Conflicts among family members	82.6 (29.65)	0.728	
Difficulty making decisions together as a family	82.6 (28.10)	0.854	
Difficulty solving family problems together	82.0 (30.53)	0.832	
Stress or tension among family members	77.3 (27.72)	0.623	

The total number of subjects is 44.
Abbreviations: I-T co = item-total correlation, Cb's A = Cronbach's alpha.

Discussion

This study demonstrates the preliminary reliability of the translated Malay version of the PedsQL™ Family Impact Module for measuring the impact of CWD on the parents and family. The results showed good internal consistency, with Cronbach's alpha values ranging from 0.730 to 0.895 across 6 domains. It is suggested that a Cronbach's alpha of 0.7 or 0.8 indicates excellent internal consistency (6–8). Varni et al. (4) found that all scales exceeded the minimum reliability standard of 0.7. This finding is similar to the findings of a study conducted among 97 parents of children with sickle cell disease in Wisconsin in the United States (9). However, a similar study conducted among 66 Brazilian families of outpatient children diagnosed with malignant neoplasm and receiving chemotherapy found that the internal consistency for some items (emotional, communication, and worry) was less than 0.7 (10).

Our study showed that the highest Cronbach's alpha was for the family relationship domain (0.895). Similarly, Varni et al. (4) and Panepinto et al. (9) found that the Cronbach's alpha for the family relationship domain had the highest score (Cronbach's alphas of 0.97 and 0.96, respectively). Our findings were also consistent with other studies (9,10) that found the lowest Cronbach's alpha value for the communication domain. In general, the corrected item-total correlation for all domains was acceptable, with the exception of the item "do not understand my family situation". This finding could be explained by the difference between this item and the other 2 items in the communication domain, which directly indicate communicable words such as "talk" and "tell".

The mean score of all functions in our study was higher than those reported by Varni et al. (4) for the outpatient samples, except for the cognitive function. This finding indicates that the caregivers in our study functioned better than those in the previous study. This finding might be explained by a possibly higher acceptance among the Malay population of the fate of having children with chronic illnesses, although the facilities available in Malaysia are relatively limited.

A limitation of this cross-sectional study with no comparison group is that it may not be sufficient to fully demonstrate the external validity of the questionnaire. Because the caregivers were all from a single state within Peninsular Malaysia, it is uncertain whether the findings can be generalised to the rest of Malaysia. However, the standard Malay language was used, and it is likely that there are no major differences with other states in Malaysia. The Malay language is also used in other countries in Southeast Asia (Indonesia, Brunei, and Singapore). Therefore, this Malay version may benefit a large number of children and caregivers in this region. Due to time constraints, the test–retest, which demonstrates the stability of the information, was not conducted. Therefore, we recommend that future studies should compare the findings with normal, healthy controls and among different types of disabilities, and that a test–retest analysis be conducted with a larger sample size.

Conclusion

The Malay version of the PedsQL™ Family Impact Module has excellent internal consistency. However, future studies with a larger sample size are necessary before it can be recommended as a tool to measure the impact of disabilities on Malay-speaking Malaysian families.

Acknowledgement

We would like to acknowledge all caregivers involved in the study, Kelantan Foundation for the Disabled, Universiti Sains Malaysia Research University grant 1001/PPSP/812049, Division of Industry and Community Network, Universiti Sains Malaysia, and those involved in the Sustainable Optimal Service for OKU.

Authors' Contributions

Conception and design: AAR, AAA
Obtaining of funding: AAR, WPWI, MII
Collection and assembly of the data: AAR, SH, NHA, MII, AO
Analysis and interpretation of the data: AAR, MKI
Drafting of the article: AAR, NM
Critical revision and final approval of the article: HVR
Statistical expertise: MKI
Administrative, technical, or logistic support: AAR, WPWI,MII

Correspondence

Dr Azriani Ab Rahman
MD (USM), MMed Community Medicine (Family Health) (USM)
Department of Community Medicine
School of Medical Sciences
Universiti Sains Malaysia Health Campus
16150 Kubang Kerian
Kelantan, Malaysia
Tel: +609-7676 635
Fax: +609-767 6654
Email: azriani@kb.usm.my

References

1. Dhar RL. Living with a developmentally disabled child: Attitude of family members in India. *Soc Sci J*. 2009;**46(4)**:738–755.

2. Wallander JL, Varni JW. Effects of pediatric chronic physical disorders on child and family adjustment. *J Child Psychol Psychiatry*. 1998;**39(1)**:29–46.

3. Bumin G, Gunal A, Tukel S. Anxiety, depression and quality of life in mothers of disabled children. *SDU Tip Fak Derg*. 2008;**15(1)**:6–11.

4. Varni JW, Sherman SA, Burwinkle TM, Dickinson PE, Dixon P. The PedsQL™ Family Impact Module: Preliminary reliability and validity. *Health Qual Life Outcomes*. 2004;**2**:55.

5. Naing L, Winn T. Sample size calculation for studies of internal consistency reliability (Cronbach's alpha). Available from: http://www.kck.usm.my/jpm/index.php?option=com_docman&task=cat_view&gid=42.

6. Nunally J, Bernstein I. *Psychometric theory*. 3rd ed. New York (NY): McGraw-Hill; 1994.

7. Ware JE, Snow KK, Kosinski M, *Gandek B. SF-36 health survey: Manual and interpretation guide*. Boston (MA): The Health Institute, New England Medical Center; 1993.

8. Warschburger P, Landgraf JM, Petermann F, Freidel K. Health-related quality of life in children assessed by their parents: Evaluation of the psychometric properties of the CHQ-PF50 in two German clinical samples. *Qual Life Res*. 2003;**12(3)**:291–301.

9. Panepinto JA, Hoffmann RG, Pajewski NM. A psychometric evaluation of the PedsQL™ Family Impact Module in parents of children with sickle cell disease. *Health Qual Life Outcomes*. 2009;**7**:32.

10. Scarpelli AC, Paiva SM, Pordeus IA, Varni JW, Viegas CM, Allison PJ. The Pediatric Quality of Life Inventory (PedsQL™) family impact module: Reliability and validity of the Brazilian version. *Health Qual Life Outcomes*. 2008;**6**:35.

Original Article

An Observational Study of Blood Glucose Levels during Admission and 24 Hours Post-Operation in a Sample of Patients with Traumatic Injury in a Hospital in Kuala Lumpur

Rahmat Harun @ Haron[1, 2], Musa Kamarul Imran[3], Mohammed Saffari Mohammed Haspani[1]

Submitted: 24 Oct 2009
Accepted: 25 Oct 2010

[1] *Department of Neurosurgery, Hospital Kuala Lumpur, Bangunan IKTAR, Jalan Pahang, 50586 Kuala Lumpur, Malaysia*

[2] *Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia*

[3] *Department of Community Medicine, School of Medical Sciences, Universiti Sains Malaysia Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia*

Abstract

Background: **Traumatic brain injury (TBI) has been associated with an acute stress response mediated by the sympathoadrenomedullary axis, which can be assessed by measuring blood glucose level.**

Methods: **This prospective observational study was conducted for a year in 2007 among 294 patients who had been treated for TBI in Hospital Kuala Lumpur. Patients fulfilling the set criteria were recruited into the study and data, including blood glucose level and Glasgow Outcome Score at 3-month follow-up, were collected.**

Results: **294 patients were included in the study: 50 females (17.0%) and 244 males (83.0%). The majority of cases were young adult patients (mean age of 34.2 years, SD 13.0). The mean blood glucose level during admission and post-surgery were 6.26 mmol/L (SD 1.30, *n* = 294) and 6.66 mmol/L (SD 1.44, *n* = 261), respectively. Specifically, the mean admission glucose level associated with mild TBI was 5.04 mmol/L (SD 0.71); moderate TBI, 5.78 mmol/L (SD 1.02); and severe TBI, 7.04 mmol/L (SD 1.18). The mean admission glucose level associated with a poor outcome in patients with isolated TBI was 6.98 mmol/L (SD 1.21). Patients with admission glucose of 5.56 mmol/L (SD 1.21) were more likely to have a favourable outcome.**

Conclusion: **Mild, moderate, and severe TBI were associated with an increase in blood glucose levels during admission, and the mean increase in glucose levels is based on the severity of the isolated TBI. Surgical intervention did not cause further significant changes in blood glucose levels. Patients with isolated TBI and minimal increases in blood glucose levels were more likely to have a favourable outcome.**

Keywords: *blood glucose, Glasgow Outcome Scale, surgery, trauma, traumatic brain injury, trauma severity index*

Introduction

Hyperglycaemia is an important parameter in the prediction of outcomes in traumatic brain injury, TBI (1–3). Studies have assessed the various levels of blood glucose throughout the course of TBI, including at the time of admission, during the course of acute treatment, and during follow-up (4–7). Hyperglycaemia in severe TBI has been associated with detrimental neurological outcomes (8), especially in patients with admission glucose levels of more than 200 mg/dL (11.0 mmol/L). Higher blood glucose levels, that is, more than 300 mg/dL (16.6 mmol/L), are almost always associated with 100% mortality in paediatric TBI (5).

TBI has been associated with an acute stress response mediated by the sympathoadrenomedullary axis, which can be reflected in an increase in blood glucose level; however, whether isolated TBI by itself results in an increase in glucose level has not been resolved in previous studies. In these studies, the focus was often on various levels of severe head

injury in the presence of other major extracranial injuries (7,9). Chiolero et al. (10) studied plasma cortisol, glucagon, insulin, glucose, free fatty acid, urinary nitrogen, and catecholamine responses in severely traumatised patients. Patients were grouped according to the following classification: severe isolated head injury, multiple injuries combined with severe head injury, and multiple injuries without head injury. The authors found that isolated head injury stimulated increases in the secretion of catabolic counter-regulatory hormones even in the absence of additional non-cranial major injury.

Animal studies have also shown that moderate and severe brain injury could induce increases in blood glucose. He et al. (11) found that the blood glucose increased at different times, and this increase correlated with the severity of brain injury in rats. A marked increase in blood glucose was observed despite high levels of insulin after TBI. By investigating levels of glucose and lactate in cats after 1 hour of simulating direct trauma (concussion) to the brain, Yang et al. (12) found that lactate concentration was increased by almost 3-fold in the cortical regions directly underneath the traumatised area when compared with the control subjects. Thus, TBI can cause derangements in brain energy metabolism.

A study by Young et al. (13) revealed that the sympathetic nervous system works together with the adrenal medullary axis in producing a response to acute severe injury. In their animal study, the authors found reductions in circulating norepinephrine and other catecholamines after the removal of stress. Elevated circulating norepinephrine and other catecholamines in response to acute severe injury play important roles in the survival of injured animals. Elevated catecholamines, which occur during stress responses, induce hepatic gluconeogenesis and result in hyperglycaemia. Stover et al. (14) conducted a prospective randomised controlled study in Sprague Dawley rats and found that norepinephrine elevates extracellular glucose in the injured brain more than in arterial blood and further aggravates post-traumatic oedema formation without changing the lactate concentration. Thus, there is a possibility that norepinephrine facilitates endothelial glucose transport in addition to passive entry, depending on concentration and pressure via a damaged blood-brain barrier.

In a human study, Yang et al. (15) reported that blood glucose and catecholamines were significantly elevated; these levels correlated to the severity of the head injury. The increase in blood glucose was related to the elevation in norepinephrine and epinephrine in response to the stress induced by the head injury. He found that 90% of patients with admission blood glucose levels of 9.6 mmol/L or greater died within 1 month post-injury. Thus, admission blood glucose could be a significant predictor of outcome.

The significant increase in blood glucose has been investigated in medical comorbidities (16,17). Wong et al.'s cohort study of stroke patients in 2008 (17) showed that mean glucose levels on admission remained stable at 6.0 mmol/L in patients with ischaemic stroke without diabetes until at least 48 hours after the stroke. This would suggest that ischaemic injury alone does not raise the blood sugar level on admission. Wintergest et al. (18) showed that increased glucose variability in patients admitted to the Paediatric Intensive Care Unit is highly associated with increased morbidity and mortality.

However, in TBI, the increase in blood glucose level is always complicated by other factors. Walia et al. (19) suggested that hyperglycaemia is more strongly predictive of the outcome of 338 patients with head injury as compared with mean arterial blood pressures. When both factors were included in a regression analysis, each factor was independently associated with mortality; however, there was a stronger relationship between blood glucose and mortality than between mean arterial blood pressure and mortality. Vogelzang et al. (20) conducted an analysis on trauma and non-trauma patients and found that hyperglycaemia influenced the outcome, and that the relationship between hyperglycaemia and mortality was more pronounced in trauma patients. Overall, hyperglycaemia correlates better with mortality in trauma patients. Takanashi et al. (21) also looked retrospectively at the clinical course of patients with head injury, which have been grouped into various categories on admission. Patients with severe head injury had higher serum glucose levels, at 11.1 mmol/L (SEM 0.2), compared with patients with moderate head injury, at 9.5 mmol/L (SEM 0.2). Patients with admission glucose levels greater than 13.3 mmol/L had 100% mortality in their study, suggesting that hyperglycaemia on admission could be a significant indicator and potent predictor of head injury severity.

The demographic pattern of brain injury severity has also not been completely elucidated. Studies have reported that females are more likely to have poorer outcomes in severe TBI (22,23); however, epidemiology studies do not support

these observations (24). The age of the patient has also been shown to predict the outcome of TBI due to various factors, such as diminished autoregulatory mechanisms. It is well known that middle-aged men and women, who are also the most productive members of the countries in which they live, sustain the highest rates of head injury and the most severe types of head injury. As a result, head injury and its associated morbidity and mortality create additional economic burden for both the health care system and the country as a poorer outcome could result in significant health care resource needs. The closest description that has been published in Malaysia is the first preliminary report of the National Trauma Database. In this document, the authors reported that there were 120,000 trauma cases per year admitted to a local hospital; however, they did not mention an association between TBI and blood glucose as a stress response (25,26). Another study by Czonyska et al. (27) found that age-related declines in cerebrovascular autoregulation contribute to the relationship between age and outcome in patients following head injury. A demographic study showed that the outcome for head injury is worse as patients get older despite a better Glasgow Coma Scale (GCS) upon admission, which may or may not be a direct reflection of glucose levels on admission (27). Epidemiology studies on gender difference in outcomes after brain injury are limited; however, animal studies revealed higher fatality rates among females (14, 32). Krauss et al. (23) found that females were at least 1.5 times more likely to have poorer outcomes as compared with male. Farace et al. (24) conducted a meta-analysis to investigate possible differences in TBI according to gender. They found that outcomes were worse in women as compared with men in 85% of the measured variables, although this conclusion was limited due to the fact that only a few of the most published reports described gender differences in their outcome. The study may not adequately reflect the possible differences in outcome based on gender in patients with isolated head injury.

This study aims to establish an association between different types of isolated head injury and increased blood glucose levels among those with an isolated head injury without any major extracranial cause. It was hypothesised that higher blood glucose levels would be associated with poorer outcomes in isolated closed TBI and that, as a result, admission blood glucose level could be used as a predictor of outcome among patients with isolated TBI.

Subjects and Methods

The primary objective of this study was to determine the differences in blood glucose levels on admission and 24 hours post-operative intervention in a population of adult patients (age 18–65 years) with TBI who were admitted to Hospital Kuala Lumpur and their outcome 3 months after head injury.

This was an observational, prospective cohort study on TBI patients who were admitted to Department of Neurosurgery, Hospital Kuala Lumpur, and registered under the Neurosurgical Head Injury Registration Book from 1 January 2007 until 31 December 2007, with follow-up until 31 March 2008. This study was approved by the local institution and registered under the National Medical Research Register (research protocol ID NMRR-09-265-3254). The inclusion criteria were patients with non-penetrating TBI aged between 18 and 65 years old. Exclusion criteria were as follows: patients who fulfilled the clinical criteria for brain death upon arrival, had other major extra cranial injuries, died within 24 hours of admission, were known to have a history of diabetes mellitus, or were on long-term steroid therapy. Data were collected using a questionnaire; all of the necessary details of the patient were recorded. The selected patients were cross-checked with the Neurosurgical Head Injury Registration Book (2007) for the purpose of follow-up and collecting up-to-date information by tracing the clinical notes to add further details to the questionnaire. Data collected include patients' demography, description of injuries, computed tomography (CT) findings, Glasgow Coma Scale, blood glucose level during admission and 24 hours post-surgical intervention, and Glasgow Outcome Score. Statistical analyses (mean, standard deviation, and standard error of mean, independent *t* test, and paired *t* test) were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL). The level of statistically significant correlations was set at 0.05 in a 2-tailed fashion.

Results

Between 1 January 2007 and 31 December 2007, there were more than 50 000 trauma cases admitted to Hospital Kuala Lumpur; however, only 1152 cases were registered in the Neurosurgical Head Injury Registration Book, Department of Neurosurgery, Hospital Kuala Lumpur. Only 294 patients who fulfilled the set criteria were recruited in the study.

The mean age of the study sample was 34.2 years old (SD 13.0). The median age of the patients was 31 years old, with a mode of 22. Approximately 75% of the study participants were 43 years of age or younger. Of the 294 cases included in the study, 50 patients (17.0%) were females and 244 (83.0%) were males. Males and females were similarly distributed when categorised according to the admission GCS. The most well-represented ethnic group in the study was Malay (n = 157, 53.4%), followed by Chinese (n = 61, 20.7%), Indian (n = 43, 14.6%), and other races (n = 33, 11.2%). Most of the subjects enrolled in the study were Malaysian (n = 233, 79.2%) while the rest were from other countries. The majority of patients included in the study were either directly admitted through the Emergency Department (n = 170, 57.8%) or as referrals from district hospitals (n = 117, 39.8%). Almost all patients treated in Hospital Kuala Lumpur were involved in road traffic accidents (n = 269, 91.5%). A considerably smaller proportion of the sample were involved in falls (n = 20, 6.8%) or assaults (n = 5, 1.7%).

Patients with a GCS of 7 were highly represented in our sample (n = 69, 23.5%). Mean GCS upon admission was 9.28 (SD 2.53) There was no patient in the study sample with a GCS of less than 5. The majority of patients had severe head injury (n = 140, 47.6%), followed by moderate (n = 104, 35.4%) and mild head injury (n = 50, 17.0%). The majority of CT brain scans (n = 274, 93.2%) had the following findings: most cases were diagnosed with subdural haemorrhage (n = 134, 45.6%), followed by extradural haemorrhage (n = 82, 27.9%) and intraparenchymal haemorrhage/contusion (n = 78, 26.5%). Only 6.8% of the patients were without any findings on imaging.

The mean glucose level on admission was 6.26 mmol/L (SD 1.30) in a bell-shaped distribution. A similar bell-shaped distribution was found for the blood glucose levels of patients (n = 261, 88.8%) within 24 hours after surgical intervention, with a mean of 6.66 mmol/L (SD 1.44). Most of the patients underwent either major operation (n = 242, 82.3%) or minor operation (n = 19, 6.5%). Only a small number of patients were treated conservatively (n = 33, 11.2%).

The highest frequency of cases included patients with severe disability or a Glasgow Outcome Score of 3 (n = 111, 37.9%), followed by moderate disability (n = 82, 28.0%) and good recovery (n = 64, 21.8%). The lowest frequency was seen in patients with a Glasgow Outcome Score of 1 (dead, n = 6, 2.0%).

Table 1 shows details concerning the sample population with respect to the severity of the isolated TBI. Sex distribution, mean age, GCS, CT scan brain findings, serum glucose levels on admission and within 24 hours after operative intervention, and neurological outcome are discussed below.

Table 1: Analysis of demographic and clinical data according to the severity of traumatic brain injury

Domains	Mild	Moderate	Severe
No. of cases	50	104	140
No. according to sex (male/female)	42/8	88/16	114/26
Age, in mean (SD) years	26.4 (8.6)	31.1 (10.3)	39.3 (14.0)
Glasgow Coma Scale, in mean (SD) score	13.2 (0.4)	10.4 (1.3)	7.0 (0.8)
Computed tomography findings			
Extradural haemorrhage	29	48	5
Subdural haemorrhage	1	42	91
Intraparenchymal haemorrhage/contusion	20	14	44
Glucose level, in mean (SD) mmol/L			
Admission	5.04 (0.71)	5.78 (1.02)	7.04 (1.18)
24 hours post-surgery	5.08 (0.71)	5.92 (1.02)	7.46 (0.18)
Outcome[a]			
Favourable	50	80	16
Unfavourable	-	24	123

[a] One case in the severe group was missing during follow-up (admission cases = 294, follow-up cases = 293).

Sex distribution was similar in the group stratified by the severity of isolated TBI (mild, moderate, and severe according to GCS). There was no significant difference in age among the patients with differing severities of isolated TBI.

Patient with severe head injury had significantly higher admission and post-operative glucose levels than those with mild and moderate head injury ($P < 0.001$). Within the group themselves, the differences in glucose values were not significant. Further analysis using a paired *t* test showed that there was no significant difference ($P < 0.01$) in the admission glucose level (6.26 mmol/L, SD 1.3) when compared with the mean glucose level taken 24 hours after surgical intervention (6.66 mmol/L, SD 1.4).

Severe head injury was associated more with subdural haemorrhage and intraparenchymal haemorrhage compared with extradural haemorrhage. There was no relationship between the different types of CT brain scan findings and increased blood glucose levels. In the moderate head injury group with slightly elevated mean blood glucose levels, it seems that mild elevations can be attributed to any pathology. Subdural haemorrhage had the highest number of patients in severe head injury with significant increases in mean blood glucose level. Extradural haemorrhage did not significantly increase the blood glucose level in the severe head injury group.

Further analysis looking at the patients' outcomes was performed accordingly (Table 2). Both glucose levels on admission and within 24 hours post-surgery were analysed based on the Glasgow Outcome Score at 3-month follow-up of the patients.

Discussion

Our findings are similar to that of the National Trauma Database Report (26), in which the majority of the population involved in trauma were between 15 and 24 years of age (30.2%). Similar to other studies (24,26), we also found that males were more likely to be involved in TBI; in the National Trauma Database Report (26), 83.5% were males. Although our data showed a male-to-female ratio of 5:1, this study found that the genders were almost equally distributed in terms of admission Glasgow Coma Score. The ethnic group distribution in this study is also comparable with in the National Trauma Database Report (26): majority of patients are Malays, 53.4% versus 58%, followed by Chinese, 20.7% versus 22.5%, and Indians, 14.6% versus 15.6%. With respect to the distribution by gender, race and nationality, the study is comparable with the national data (25,26). Thus, this study may be representative of the demographic involved in isolated TBI in our country.

In this study, there was a significant difference in admission blood glucose level with regard to the different severity of isolated TBI. Severe head injury was associated with more than 1 mmol/L elevation in blood sugar in comparison to both mild and moderate head injury. However, the difference in mean blood sugar level between mild and moderate was not more than 1.0 mmol/L. Glucose levels 24 hours after operation also showed similar differences among the group; only severe head injury patients were elevated by more than 1 mmol/L compared with the other groups, while the different between mild and moderate

Table 2: Analysis of the patients' outcomes and blood glucose levels

Outcome	No. of subjects	Glucose level (mmol/L)	
		Admission	24 hours post-surgery
Glasgow Outcome Score (GOS)			
5: Dead	6	7.9 (1.96)	8.5 (1.96)
4: Persistent vegetative state	30	7.9 (1.10)	8.5 (1.10)
3: Severe disability	111	6.7 (1.05)	7.0 (1.05)
2: Moderate disability	82	5.9 (0.91)	6.1 (0.91)
1: Good recovery	64	5.0 (0.8)	5.0 (0.8)
Categorisation into two groups			
Favourable outcome (GOS 1–3)	147	5.5 (1.21)	5.7 (1.21)
Poor outcome (GOS 4–5)	146	7.0 (1.21)	7.4 (1.21)

Data are expressed in mean (SD).

was not more than 1.0 mmol/L. This could mean that the level of blood glucose corresponds to the severity of isolated TBI. Other authors have found similar findings that admission blood glucose level reflects the severity of the TBI (8,20,21,28), specifically the severity of severe head injury (2,7,19). Lam et al. (28) found that the mean admission blood glucose level was 10.7 mmol/L (SEM 0.4) in the severe head injury group, whereas the mean admission blood glucose level for mild head injury was 7.2 mmol/L (SEM 0.4). Rovlias et al. (8) reported a mean admission blood glucose level of 11.3 mmol/L (SEM 0.2) in the severe head injury group, whereas the mean admission blood glucose level for moderate head injury was 9.1 mmol/L (SEM 0.3). Both authors (8,28) documented slightly higher figures compared with the findings in our study.

With regard to the differences in blood glucose levels between the groups (admission versus 24 hours post-surgery), only those with severe TBI had significant but mild (less than 0.5 mmol/L) elevations in mean blood glucose levels. In the mild and moderate head injury group, there was no significant difference in mean glucose levels on admission compared with 24 hours after surgery. Our finding that there was no difference in glucose levels between admission and 24 hours post surgery, but that higher mean glucose levels were associated with poorer outcome, is similar to the findings of other authors (7,28). These findings imply that an isolated traumatic injury can independently and significantly increase blood glucose levels depending upon injury severity; thus, blood glucose level may be an important prognostic factor for predicting outcome among patients with TBI.

With respect to whether there is a relationship between glucose levels at admission or 24 hours post-surgery and the outcome, our study found that glucose levels were significantly associated with outcomes among patients with TBI (Table 2). For glucose levels below 6.0 mmol/L, patients had a better outcome in relation to Glasgow Outcome Score at the 3-month follow-up in the clinic (independent *t* test, $P < 0.01$). Higher blood glucose levels of the patient upon admission (more than 7.9 mmol/L) were associated with poorer outcomes; however, this association was not significant ($P = 0.98$). This pattern was also consistent for the relationships between a mean blood glucose level of 8.5 mmol/L within 24 hours post-surgery and poor outcomes: dead, $P = 0.92$, and persistent vegetative state, $P = 0.94$ (Table 2). Walia et al. (19) investigated the relationship between blood pressure, blood glucose concentration, and outcome following severe head injury. They found that both mean arterial pressure (MAP) and blood glucose are related to mortality in a linear fashion ($P < 0.0001$). Their regression analysis showed that each of the studied factors was an independent predictor of mortality. The relationship between blood glucose and mortality was much stronger than the relationship between MAP and mortality. By grouping the patients together according to the lowest MAP, hyperglycaemia was associated with increasing mortality within each group ($P < 0.0001$).

Chiaretti et al. (29) conducted a study among children with head injury and found a similar relationship as the one found in our study: hyperglycaemia occurred more frequently in children with severe head injury than in those with mild and moderate head injury. Their conclusion was that persistent hyperglycaemia beyond 24 hours after injury appears to be an important negative prognostic factor. Cochran et al. (5) found that hyperglycaemia can be an independent predictor of outcomes in paediatric TBI. Patients with admission blood glucose of lower than 7.5 mmol/L were more likely to live, and those with blood glucose of greater than 14.8 mmol/L were more likely to die. Admission blood glucose levels of greater than 16 mmol/L were associated with 100% mortality. An earlier study (30) demonstrated that head injury patients with persistent hyperglycaemia (defined as plasma glucose of more than 15 mmol/L in the study) had grave outcomes (mean time of survival was 2.1 days, SD 1.4). Looking retrospectively at intensive care patients with severe head, Jeremitsky et al. (31) found that hyperglycaemia was an independent predictor of severity and outcome, that is, the higher the score, the poorer the outcome. A study by Salim et al. (2) found that persistent hyperglycaemia may be an independent risk factor for mortality with an odds ratio of 4.91 (95% CI 2.88–8.56, $P < 0.0001$). They defined persistent hyperglycaemia as an average daily blood glucose level of equal to or greater than 8.3 mmol/L. Beek et al. (1) found that the predictive value of blood glucose for predicting outcome among those with TBI was strongest for increasing blood levels of glucose (odds ratio 1.7, 95% CI 1.54–1.83).

Kinoshita et al. (32) provided a possible explanation for why early hyperglycaemia might affect outcomes in patients with isolated TBI. In their study, the authors created a rat model of moderate TBI and allocated rats to

1 of 4 treatment groups: early group given dextrose injection 5 minutes after trauma, delayed group given dextrose injection 4 and 24 hours after trauma, and a control group. They measured contusion areas and volumes, as well as frequency of myeloperoxidase immunoreactive polymorphonuclear leukocytes (PMNLs). They found that acute hyperglycaemia did not significantly affect total contusion volume but did increase contusion area, as well as enhanced accumulation of PMNLs within the contusion area. Delayed induced hyperglycaemia did not produce the same result. The result of their study indicated that early or acute hyperglycaemia aggravates histopathological outcomes and increases the accumulation of PMNLs, leading to worsening of outcomes by enhanced secondary injury, including inflammation.

Instead of using exogenous glucose, Stover et al. (14) used catecholamines (norepinephrine and dopamine) to induce hyperglycaemia, as catecholamines increased hepatic gluconeogenesis in their study in TBI Sprague Dawley rats. They found that norepinephrine and dopamine increased arterial blood glucose, thereby significantly increasing pericontusional cortical glucose and lactate concentrations, but did not increase extracellular lactate concentrations. This leads to aggravation of underlying post-traumatic oedema and further worsens the outcome among patients with TBI. In addition to possibly increasing facilitated endothelial glucose transport, the elevated extracellular to blood glucose ratio suggests passive concentration-dependent and pressure-dependent entry via a damaged blood-brain barrier. This might contribute to the observed reversible increase in extracellular glucose, meaning that the higher the blood glucose level with TBI, the poorer the outcome. Kinoshita et al. (32) has shown earlier that the increased contusion area in vivo also indicates that hyperglycaemia in TBI might also require control treatment to improve the outcome of head injury patients.

Inference of reversibility of the increase in extracellular glucose concentration, which occurs as a result of the damaged blood–brain barrier and leads to poorer outcome in head injury patients, has given others encouragement to find other proof for this reversibility. Zygun et al. (9) further tested the hypothesis that blood glucose levels are associated with brain tissue pH and that the correction of hyperglycaemia would result in improvement of brain tissue pH. They used 428 glucose measurements with pH monitoring and a linear generalised estimating equation model to assess the relationship. They found that the difference between baseline readings of brain tissue pH and glucose with subsequent readings of brain tissue pH and glucose of less than 11.1 mmol/L was not significant (P = 0.29). If the change in blood glucose had been large, there would have been a suggestion of improvement in the brain tissue pH. Thus, there is potential proven benefit for treating hyperglycaemia in TBI.

Limitations

The primary limitation of this study was the number of patients recruited. The required sample size, according to calculation, was 445 patients; however, this study assessed only 294 patients. The sample size was small and may not have had a sufficient amount of power to detect significant differences. Furthermore, the study sample collected did not truly reflect the actual number of patients that were eligible for the study within the institution.

The number of cases in the mild head injury group was relatively smaller compared with the number of cases in the severe head injury group. These 2 groups were the groups of interest for determining significant differences in glucose concentration. With such small numbers in each group, further interpretations to the relationship of TBI with different CT scan findings were unsuccessful.

In addition, the measurement of blood glucose level was a source of bias. A standardised method for measuring glucose levels was used consistently in patients who were admitted and underwent surgical intervention. Patients who were treated conservatively may not have received similar lab blood work, thus introducing a source of bias.

Conclusion

This study demonstrated a significant difference in blood glucose levels among patients with isolated TBI. Severe TBI caused a significant rise in blood glucose levels during admission, and the mean glucose level increased according to the severity of the isolated TBI. Surgical intervention did not cause any further significant changes in blood glucose levels, suggesting that isolated TBI alone may cause a significant elevation in blood glucose levels. In our study, higher blood glucose levels upon admission after isolated TBI were associated with poorer outcomes for patients.

This study also showed that hyperglycaemia is an important independent predictor of outcome. Blood glucose levels may be a good independent

predictor of outcome in TBI. There is evidence to suggest that tight control of blood glucose in patients with TBI may improve outcomes for these patients. Future research addressing this specific question is warranted.

Authors' Contributions

Conception and design, collection and assembly of the data, drafting of the article: RHH
Analysis and interpretation of the data, statistical expertise: KIM
Provision of study materials or patients, critical revision and final approval of the article, administrative, technical, or logistic support: MSMH

Correspondence

Dr Rahmat Harun @ Haron
MSurg Neurosurgery (USM)
Department of Neurosurgery
Hospital Kuala Lumpur
Bangunan IKTAR, Jalan Pahang
50586 Kuala Lumpur
Malaysia
Tel: +603-2615 5190
Fax: +603-2698 2598
Email: rahmat865@yahoo.com

References

1. Van Beek JG, Mushkudiani NA, Steyerberg EW, Butcher I, McHugh GS, Lu J, et al. Prognostic value of admission laboratory parameters in traumatic brain injury: Results from the IMPACT study. *J Neurotrauma*. 2007;**24(2)**:315–328.

2. Salim A, Hadjizacharia P, Dubose J, Brown C, Inaba K, Chan LS, et al. Persistent hyperglycemia in severe traumatic brain injury: An independent predictor of outcome. *Am Surg*. 2009;**75(1)**:25–29.

3. Baranov D, Neligan P. Trauma and aggressive homeostasis management. *Anesthesiol Clin*. 2007;**25(1)**:49–63.

4. Bochicchio GV, Salzano L, Joshi M, Bochiocchio K, Scalea TM. Admission preoperative glucose is predictive of morbidity and mortality in trauma patients who require immediate operative intervention. *Am Surg*. 2005;**71(2)**:171–174.

5. Cochran A, Scaife ER, Hansen KW, Downey EC. Hyperglycaemia and outcomes from pediatric traumatic brain injury. *J Trauma*. 2003;**55(6)**: 1035–1038.

6. De Salles AA, Muizelaar JP, Young HF. Hyperglycemia, cerebrospinal fluid lactic acidosis, and cerebral blood flow in severely head-injured patients. *Neurosurgery*. 1987;**21(1)**:45–50.

7. Young B, Ott L, Dempsey R, Haarck D, Tibbs P. Relationship between admission hyperglycemia and neurologic outcome of severely brain-injured patients. *Ann Surg*. 1989;**210(4)**:466–472; discussion 472–473.

8. Rovlias A, Kotsou S. The influence of hyperglycemia on neurological outcome in patients with severe head injury. *Neurosurgery*. 2000;**46(2)**:335–342; discussion 342–343.

9. Zygun DA, Steiner LA, Johnston Aj, Hutchinson PJ, Al-Rawi PG, Chatfield D, et al. Hyperglycemia and brain tissue pH after traumatic brain injury. *Neurosurgery*. 2004;**55(4)**:877–881; discussion 882.

10. Chiolero R, Schutz Y, Lemarchand T, Felber JP, de Tribolet N, Freeman J, et al. Hormonal and metabolic changes following severe head injury or noncranial injury. *JPEN J Parenter Enteral Nutr*. 1989;**13(1)**: 5–12.

11. He ZH, Zhi XG, Sun XC, Tang WY. [Correlation analysis of increased blood glucose and insulin resistance after traumatic brain injury in rats]. *Nan Fang Yi Ke Da Xue Xue Bao*. 2007;**27(3)**:315–317.

12. Yang MS, DeWitt DS, Becker DP, Hayes RL. Regional brain metabolite levels following mild experimental head injury in the cat. *J Neurosurg*. 1985;**63(4)**: 617–621.

13. Young JB, Fish S, Landsberg L. Sympathetic nervous system and adrenal medullary responses to ischemic injury in mice. *Am J Physiol*. 1983;**245(1)**:E67–E73.

14. Stover JF, Sakowitz OW, Thomale UW, Kroppenstedt SN, Unterberg AW. Norepinephrine-induced hyperglycemia does not increase cortical lactate in brain-injured rats. *Intensive Care Med*. 2002;**28(10)**:1491–1497.

15. Yang SY, Zhang S, Wang ML. Clinical significance of admission hyperglycemia and factors related to it in patients with acute severe head injury. *Surg Neurol*. 1995;**44(4)**:373–377.

16. Cely CM, Arora P, Quartin AA, Kett DH, Schein RM. Relationship of baseline glucose homeostasis to hyperglycemia during medical critical illness. *Chest*. 2004;**126(3)**:879–887.

17. Wong AA, Schluter PJ, Henderson RD, O'Sullivan JD, Read SJ. Natural history of blood glucose within the first 48 hours after ischemic stroke. *Neurology*. 2008;**70(13)**:1036–1041.

18. Wintergerst KA, Buckingham B, Gandrud L, Wong BJ, Kache S, Wilson DM. Association of hypoglycemia, hyperglycemia, and glucose variability with morbidity and death in the pediatric intensive care unit. *Pediatrics*. 2006;**118(1)**:173–179.

19. Walia S, Sutcliffe AJ. The relationship between blood glucose, mean arterial pressure and outcome after severe head injury: An observational study. *Injury*. 2002;**33(4)**:339–344.

20. Vogelzang M, Nijboer JM, van der Horst IC, Zijlstra F, ten Duis HJ, Nijsten MW. Hyperglycemia has a stronger relation with outcome in trauma patients than in other critically ill patients. *J Trauma*. 2006;**60(4)**:873–877; discussion 878–879.

21. Takanashi Y, Shinonaga M, Nakajima F. [Relationship between hyperglycemia following head injury and neurological outcome]. *No to Shinkei*. 2001;**53(1)**: 61–64.

22. Marion DW. *Traumatic brain injury*. New York: Thieme; 1999.

23. Kraus JF,Peek-Asa C, McArthur D. The independent effect of gender on outcomes following traumatic brain injury: A preliminary investigation. *Neurosurg Focus*. 2000;**8(1)**:1–7.

24. Farace E, Alves WM. Do women fare worse: A metaanalysis of gender differences in traumatic brain injury outcome. *J Neurosurg*. 2000:**93(4)**:539–545.

25. Sabariah FJ, Ramesh N, Mahathar AW. National Trauma Database (NTrD)–Improving trauma care: First year report. *Med J Malaysia*. 2008; **63(Suppl C)**:45–49.

26. Sabariah FJ, editor. *National Trauma Database May 2006 To April 2007–First Report*. Kuala Lumpur (MY): National Trauma Database (MY); 2007.

27. Czosnyka M, Balestreri M, Steiner L, Smielewski P, Hutchinson PJ, Matta B, et al. Age, intracranial pressure, autoregulation, and outcome after brain trauma. *J Neurosurg*. 2005;**102(3)**:450–454.

28. Lam AM, Winn HR, Cullen BF, Sundling N. Hyperglycemia and neurological outcome in patients with head injury. *J Neurosurg*. 1991;**75(4)**:545–551.

29. Chiaretti A, De Benedictis R, Langer A, Di Rocco C, Bizzarri C, Iannelli A, et al. Prognostic implications of hyperglycaemia in paediatric head injury. *Childs Nerv Syst*. 1998;**14(9)**:455–459.

30. Merguerian PA, Perel A, Wald U, Feinsod M, Cotev S. Persistent nonketotic hyperglycemia as a grave prognostic sign in head-injured patients. *Crit Care Med*. 1981;**9(12)**:838–840.

31. Jeremitsky E, Omert LA, Dunham CM, Wilberger J, Rodriguez A. The impact of hyperglycemia on patients with severe brain injury. *J Trauma*. 2005;**58(1)**: 47–50.

32. Kinoshita K, Kraydieh S, Alonso O, Hayashi N, Dietrich WD. Effect of posttraumatic hyperglycemia on contusion volume and neutrophil accumulation after moderate fluid-percussion brain injury in rats. *J Neurotrauma*. 2002;**19(6)**:681–692.

Special Communication

Medicalisation of Suicide

Saxby Pridmore

Submitted: 14 Apr 2011
Accepted: 22 Jun 2011

Department of Psychiatry, School of Medicine, University of Tasmania, Private Bag 27, Hobart, Tasmania, Australia 7001

Abstract

Medicalisation is the misclassification of non-medical problems as medical problems. A common form of medicalisation is the misclassification of normal distress as a mental disorder (usually a mood disorder). Suicide is medicalised when it is considered a medical diagnosis per se, when it is considered to be secondary to a mental disorder when no mental disorder is present, and when no mental disorder is present but the management of suicidal behaviour associated with distress is believed to be the sole responsibility of mental health professionals. In the West, psychological autopsies have led to the belief that all or almost all suicide is the result of mental disorder. However, there are reservations about the scientific status of such studies. The actions of psychological autopsy researchers, coroners/magistrates, police, policy writers, and grieving relatives all contribute. Medicalisation of suicide has the potential to distort research findings, and caution is recommended.

Keywords: *depression, mental health, prevention, suicide, terminology, Western world*

Introduction

In the West, suicide has been viewed differently over time. When Greece was the centre of the Western civilisation, suicide was viewed as a moral response to disgrace and an appropriate method of making a political statement. Later, throughout Europe, suicide became a legal matter, a disgraceful act, an insult to God, and a legally punishable offence. The bodies of people who had completed suicide were desecrated, they could not be buried in graveyards with others, and their estates could not be inherited by their families, but were forfeited to the state. In 1821, the influential French physician Esquirol (1) declared that suicide was a medical problem. Since about that time, throughout the West, suicide has been understood in terms of mental disorder. This paper contends that, while suicide is more common among people with mental disorder, it also occurs in people without mental disorder, and medicalisation prevents a more comprehensive view of this behaviour. Countries in Asia are now conducting important studies in this field, and the view that all suicide is due to mental disorders needs to be approached with caution.

The concepts which underpin this paper include that suicide is medicalised when any of the following apply: 1) suicide is believed to be a medical disorder per se, 2) suicide is believed to be the direct result of a medical disorder when no medical disorder actually exists, and 3) the management suicidal behaviour that is not associated with severe mental disorder is deemed to be the role and responsibility of mental health professionals.

The first circumstance can be immediately excluded because suicide is not a medical diagnosis; it is a legal finding. The second and third circumstances frequently depend on the medicalisation of distress, that is, distress is misclassified as a mental disorder. Therefore, the medicalisation of distress needs also to be examined.

Medicalisation

Medicalisation is the misclassification of non-medical problems as medical problems (2). It has been discussed over recent decades in the Western social science literature (3). The Asian literature (4), however, suggests some awareness and resistance to this process.

Van Praag (5) described medicalisation as a process by which "normal" human behaviour and experience is "re-badged" as a series of medical conditions. Chodoff (6) stated that "the human condition" is medicalised by application of a "diagnostic label to various unpleasant or undesirable feelings or behaviors" which are, in reality, "inescapable aspects of the fate of being human".

Examples include 1) shyness being classified as "social anxiety", 2) promiscuity being classified as "sexual addiction", 3) everyday worrying being

classified as "anxiety disorder", and 4) low sexual desire in females being classified as "female sexual arousal disorder".

When medicalisation occurs and a medical explanation is accepted, it follows that a treatment will be provided (7). Examples include when ordinary emotional distress is classified as psychiatric disorder and treated with psychotropic medication, and when ordinary physical conditions (such as baldness and overweight) are classified as pathological states and treated with surgery.

A number of factors prepared the way for the emergence of medicalisation. Most prominent among them are 1) the universal acceptance of a very broad definition of health, and 2) the absence of precise definitions for the terms mental health, mental disorder, and mental health problems.

The World Health Organization (WHO) defined "health" as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity". Well-being, in turn, is defined as "a contented state of being happy, healthy and prosperous" (8). Thus, the terms health and well-being are interchangeable. More recently, the WHO Commission on Social Determinants of Health (9) advocated not only for healthy lives, but also for all individuals to live a "flourishing life". Thus, very high expectations are encouraged, and a hangover following drinking or a loss of money at the races, both of which impact on happiness or well-being, could be classed as health issues needing treatment.

"Mental health" has also been described in positive, optimal terms. For example, one authority states, "In general, mentally healthy individuals value themselves, perceive reality as it is, accept its limitations and possibilities, respond to its challenges, carry out their responsibilities, establish and maintain close relationships, deal reasonably with others, pursue work that suits their talent and training, and feel a sense of fulfillment that makes the effort of daily living worthwhile" (10).

Very importantly, "mental disorder" lacks a satisfactory definition. The Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV, 11) states "... no definition adequately specifies precise boundaries for the concept of mental disorder" (p. xxx). In the absence of a definition, it provides a description which begins "...each of the mental disorders is conceptualized as a clinically significant behavioural or psychological syndrome or pattern that occurs in an individual and that is associated with present distress or disability..." (p. xxxi). This description employs vague, undefined terms including "clinically significant", "psychological syndrome", and "distress". Using this description, it is impossible to differentiate mental disorders from normal human experiences such as guilt and grief, although the man in the street and most health professionals believe a distinction can and should be made.

The category "mental health problem" has been used (and may have been invented) in Australia (12). An Australian Government publication (13) states, "A mental health problem also interferes with how a person thinks, feels, and behaves, but to a lesser extent than a mental illness. Mental health problems are more common and include the mental ill health that can be experienced temporarily as a reaction to the stresses of life". Thus, the temporary reactions to the stresses of life have, in Australia at least, been designated as forms of "ill health" and thereby, the responsibility of the mental health services.

Medicalisation was initially blamed on doctors, who were described as attempting to increase their power (and the term "medical imperialism" was coined). However, balanced views now identify many "drivers" of medicalisation (14), including drug companies who seek to sell their products (15). Other drivers include the advantages of the sick-role; Mechanic (16) described the benefits of the sick role as relief from the responsibility of caring for oneself and family, and from going to work. Other commentators believe governments encourage medicalisation as a means of dealing with difficult social problems (for example, lowering unemployment figures by placing people on sickness pensions).

The WHO has a broad view of "health" and advocates a "flourishing life". However, health departments have little influence over most of the things that foster a "flourishing life": freedom, democracy, fairness, justice, educational and employment opportunity, affordable housing and transport, et cetera. Medical practitioners and services have extended their traditional roles to remove, wherever possible, distress associated with the "human condition". The minimisation of distress is, of course, desirable; whether this should be achieved via medicalisation, which distorts some medical tenants, is a matter for debate.

This section closes with the Buddha on the ubiquity of pain in life: "Birth is painful; old age is painful; sickness is painful; death is painful; sorrow, lamentation, dejection, and despair are painful. Contact with unpleasant things is

painful; not getting what one wishes is painful" (The Sermon at Benares). A current challenge is to decide which human problems are health problems and which, if any, are not.

Distress Medicalised into Depression

The term "depression" has at least two meanings, one is colloquial and another is technical. In contemporary discussions, the term "depression" is frequently used without clarification about which meaning is intended. This is a leading contributor to medicalisation of distress.

The colloquial meaning of "depression" is low mood/spirits of any degree, and as a result of any cause. At one extreme, it can be applied when the mood is slightly lowered for a brief period, as the result of a trifling loss. At the other extreme is more severe lowering of mood, as the result of a great loss.

When used in a technical sense by mental health professionals, the term "depression" is used to refer to a mental disorder (or sickness) featuring low mood/spirit most often called major depressive disorder (MDD). This is a serious and usually recurring disorder, characterised by episodes which often last months, but which may be shortened by treatment. Early episodes of mood disorder may be triggered by unhappy events such as loss and later episodes (relapses) may occur without detectable triggering events (losses).

Most importantly, the diagnosis of MDD can only be made when, in addition to persistent depressed mood or loss of the ability to experience pleasure, other symptoms are present. For a diagnosis to be made, at least four additional symptoms are required: these include a significant change in appetite, sleep problems, agitation or retardation, loss of energy, feelings of worthlessness, inability to concentrate, and thoughts of suicide (11).

A common example of the way distress is medicalised is when an individual who is distressed by an everyday event (for example, a cheating lover) reports that he/she feels "depressed", and this is taken to indicate "depression" in the mental disorder sense, even though the other diagnostic criteria have not been satisfied. Accordingly, the sick role is granted (paid leave from work and psychotropic medication become options). The individual may not claim the sick role; it may be that well-meaning others who observe the distress, with good intentions, thrust the sick role on the individual. There may be some initial advantages to the distressed individual in the form of increased social support, but in the long term, the disadvantages of the sick role out-weigh any advantages.

A major facilitating factor in the medicalisation of distress is that the DSM-IV (11) pays no attention to the context in which symptoms occur (except in the case of bereavement). If your house burns down, your spouse runs off, and you are diagnosed with cancer, all in the same week, as long as you have five MDD symptoms for two weeks, you can be diagnosed with MDD. The making of such a diagnosis is justified (according to the DSM-IV), even though your friends believe you are dealing very well with a nasty run of bad luck. Horwitz and Wakefield (17) make this criticism in their important monograph, *The Loss of Sadness: How Psychiatry Transformed Normal Sorrow into Depressive Disorder*.

How Suicide is Medicalised

Suicide is not a medical diagnosis; it is a legal finding. The central features are that the death occurs as a result of actions taken by the deceased, and these actions were taken with the intention of causing death.

As mentioned, Esquirol (1) was influential in the medicalisation of suicide in the early 19th century (18); others describe this process commencing in the late 18th century. Our concern here, however, is with current practices.

Much Western academic writing has contributed to the medicalisation of suicide. For example, Moscicki (19) states that "a psychiatric disorder is a necessary condition for suicide to occur", and Jamison (20) states that there is "unequivocal presence of severe psychopathology in those who die by their own hand". Some authors state that a psychiatric disorder is present in 100% of cases of suicide (21,22), and estimates of above 90% are widely reported (23,24). These findings are based on psychological autopsies: evidence is gathered about the thinking and actions of the deceased, and conclusions are drawn as to whether or not a mental disorder was been present. These are retrospective studies, and there are serious reservations about their validity and reliability (25–27) and the quality of the diagnostic instruments that are used (28). Thus, the scientific quality of psychological autopsies is not proven.

Even if the methodological issues could be overcome with certainty, the possibility remains that distress may be medicalised and recorded as a mood disorder. It is reasonable to assume that

all those who complete suicide are distressed, and therefore, psychological autopsy provides the opportunity for misclassification.

Recent Asian psychological autopsy studies have provided different results. An Indian study (29) found mental disorder in less than 40% of decedents, and studies of young people in China (30,31) have found an Axis I disorder in less than 50% of decedents. A report from Korea (32) found that "the current suicide epidemic in Korea has social origins". Given the potential for psychological autopsies to medicalise distress, findings that psychiatric disorder is present in less than 50% of the deceased suggests that medicalisation of suicide is much less common in Asia than in the West.

Another opinion, based not on psychological autopsies but on historical documents and qualitative material, acknowledges that suicide is more common among those with mental disorder, but holds that suicide can, and likely frequently does occur, in the absence of mental disorder (33,34). It should be mentioned that Western sociological autopsies and reviews have provided support for social factors contributing to suicide (35–37).

In addition to the psychological autopsy studies, a range of other actions encourage the medicalisation of suicide.

Officials (coroners, magistrates, et cetera, depending on local regulations) closely examine cases of suicide for evidence of health professional negligence or neglect, and frequently make negative findings (usually considered by the involved health professionals to be unjustified). By this process, officials reinforce the view that suicide is a psychiatric phenomenon and a matter of medical responsibility. Newspapers report these findings and supplement them with additional details. The police medicalise suicide by seeking to transfer everyone they apprehend who mentions suicide into the hospital system. They are motivated by the reasonable desire to avoid the hassles associated with a death in custody.

Suicidal thoughts (whether arising out of mental disorder or non-disorder distress) are terrifying to the individual and his/her associates, leading to a rush to a place of "safety" (the hospital). This is an understandable and often appropriate response in contemporary life, but can also be viewed in the context of medicalisation.

Self-help groups, some researchers and clinicians, and policy writers promote the notion that suicide is universally the result of mental disorder, because mental disorder is potentially treatable, and this notion allows the welcome belief that a path to suicide prevention is readily available.

When suicide has occurred, family members may prefer to believe and promote the explanation that the deceased must have suffered an unrecognised or untreated mental disorder, as a means of deflecting responsibility away from the deceased and survivors.

The great disadvantage of all-suicide-is-caused-by-mental-disorder thinking is that important social, cultural, economic, and political factors, about which much might be done, are neglected in favour of the medical solution. Relevantly, the medical solution has been the focus of national suicide prevention strategies around the world, but none of these have reduced national suicide rates (38).

Another disadvantage of the medicalisation of suicide is that it leads to suicidal behaviour becoming a socially acceptable response to distress (certainly, this is the case among young people in the West). Thus, medicalisation of suicide makes suicidal responses more, rather than less, likely.

Those individuals who have a mental disorder and are at a risk of suicide should receive all possible help. At times of acute risk, they should be kept as safe as possible and the mental disorder treated. Special supervision and support may be necessary and involve admission (at times, involuntary) to the hospital. The individual who has lost all interest in food and fluid may need special treatment for malnutrition and dehydration, with a view to preserving life long enough for treatment to take effect, and emergency electroconvulsive therapy may be necessary. This is not medicalisation, but appropriate medical care.

Support can come from family, friends, clergy, teachers, and a range of people with experience of the world. However, the traditional extended family and religion currently provide less social support than formerly (certainly in the West), and scholars (39,40) describe medicalisation as compensating for this social change.

Limitations of This Paper

The limitations of this study include that it is the opinion of one individual, and as such, incorporates biases. Nevertheless, it is based on decades of clinical observations by a trained psychiatrist. It takes a rigid view on the nature of mental disorders. It conceptualises the responsibilities of mental health services as primarily the treatment of mental disorders,

while current thinking is tending to broaden these out to include mental health and mental health problems. More flexible views of the responsibilities of mental health services have been described (41).

Summary

Medicalisation is the misclassification of non-medical problems as medical problems (2). It leads to poor outcomes and distorts our understanding of phenomena. The medicalisation of distress and suicide deserves close consideration.

Suicide is a piece of behaviour that is a final common pathway out of various distressing situations/predicaments (34). One of these distressing situations/predicaments is serious mental disorder, particularly MDD, especially when the disorder is untreated or unresponsive to treatment. The distress associated with a predicament, however, may not meet the diagnostic criteria of a mental disorder. Importantly, the High Court of Australia has found that suicide "may or may not involve mental illness" (42).

Medicalisation is facilitated by the very broad WHO definition of health, and the very imprecise DSM-IV definition of mental disorder.

It is frequently unrecognised that medicalisation (for example, treating a distressed person as if they are sick as an act of kindness) is stigmatising and often disadvantageous to the development of that individual.

Suicide can be medicalised via different processes, including being considered synonymous with mental disorder, by concluding that it has been triggered by a mental disorder when no such disorder exists, and by suicidal behaviour that is not the result of mental disorder being cast as the role and responsibility of mental health professionals.

In the West, psychological autopsies have been influential in the medicalisation of suicide: they have frequently found that 100% of those who completed suicide have suffered mental disorder. The psychological autopsy method, however, has scientific limitations. In Asia, psychological autopsies have found mental disorders less commonly (often in less than 50% of cases). These differences may be attributable to greater medicalisation in the West, but other cultural factors are probably also important.

Other actors also play a role in the medicalisation of suicide, including coroners, police, self-help groups, some researchers and clinicians, policy writers, and grieving families.

Correspondence

Professor Dr Saxby Pridmore
MB, BS, BMedSc (Tasmania), DPhysio (Melbourne), MD (Tasmania), AM
Department of Psychiatry
School of Medicine
University of Tasmania
Private Bag 27, Hobart
Tasmania, Australia 7001
Tel: +61 0409 825 029
Fax: +61 03 6226 4777
Email: s.pridmore@utas.edu.au

References

1. Esquirol J. Suicide. In: Societe de Medecins et de Chirurgiens. *Dictionnaire des sciences medicales. Volume LIII*. Paris (FR): CLF Panckouke; 1812–1822.
2. Zola IK. Medicine as an institution of social control. *Sociol Rev*. 1972;**20(4)**:487–504.
3. Watters E. *Crazy like us: The globalization of the American psyche*. Melbourne (AU): Scribe Publications; 2004.
4. Lee S. Diagnosis postponed: Shenjing shuairuo and the transformation of psychiatry in post-Mao China. *Cult Med Psychiatry*. 1999;**23(3)**:349–380.
5. Van Praag HM. Nosologomania: A disorder of psychiatry. *World J Biol Psychiatry*. 2000;**1(3)**: 151–158.
6. Chodoff P. The medicalization of the human condition. *Psychiatr Serv*. 2002;**53(5)**:627–628.
7. Aho K. Medicalizing mental health: A phenomenological alternative. *J Med Humanit*. 2008;**29(4)**:243–259.
8. World Health Organization. *Constitution of the World Health Organization*. Geneva (CH): World Health Organization; 1946.
9. Commission on Social Determinants of Health. *Closing the gap in a generation: Health equity through action on the social determinants of health. Final report of the Commission on Social Determinants of Health*. Geneva (CH): World Health Organization; 2008.
10. Hales D, Hales RE. Caring for the mind: *The comprehensive guide to mental health*. New York (NY): Bantam Books; 1996.
11. American Psychiatric Association. *Diagnostic and statistical manual of mental disorders: Fourth edition: Text revision*. Washington (DC): American Psychiatric Association; 2000.
12. Australian Health Ministers. *National mental health policy*. Canbera (AU): Australian Government Publishing Service; 1992.
13. *What is mental illness*? Canberra (AU): Mental Health and Workforce Division of the Australian Government Department of Health and Ageing; 2007.

14. Conrad P. *The medicalization of society: On the transformation of human conditions into treatable disorders*. Baltimore (MD): John Hopkins University Press; 2007.

15. Doran E, Henry D. Disease mongering: Expanding the boundaries of treatable disease. *Intern Med J*. 2008;**38(11)**:858–861.

16. Mechanic D. *Medical sociology: A selective view*. New York (NY): Free Press; 1968.

17. Horwitz AV, Wakefield JC. *The loss of sadness: How psychiatry transformed normal sorrow into depressive disorder*. New York (NY): Oxford University Press; 2007.

18. Watts JR, editor. *From sin to insanity: Suicide in early modern Europe*. Cornell (NY): Cornell University Press; 2004.

19. Moscicki E. Identification of suicide risk factors using epidemiologic studies. *Psychiatr Clin North Am*. 1997;**20(3)**:499–517.

20. Jamison KR. *Night falls fast: Understanding suicide*. New York (NY): Vintage Books; 1999.

21. Dorpat T, Ripley H. A study of suicide in the Seattle area. *Compr Psychiatry*. 1960;**1**:349–359.

22. Ernst C, Lalovic A, Lesage A, Seguin M, Tousignant M, Turecki G. Suicide and no axis I psychopathology. *BMC Psychiatry*. 2004;**4**:7.

23. Bertolote J, Fleischmann A, De Leo D, Wasserman D. Psychiatric diagnoses and suicide: Revisiting the evidence. *Crisis*. 2004;**25(4)**:147–155.

24. Scocco P, Marietta P, Tonietto M, Dello Buono M, De Leo D. The role of psychopathology and suicide intention in predicting suicide risk: A longitudinal study. *Psychopathology*. 2000;**33(3)**:143–150.

25. Selkin J, Loya F. Issues in the psychological autopsy of a controversial public figure. *Prof Psychol*. 1979;**10(1)**:87–93.

26. Ogloff JRP, Otto RK. Psychological autopsy: Clinical and legal perspectives. *St Louis Univ Law J*. 1993;**37**:607–646.

27. Tu WQ, Zhao H. Psychological autopsy and its limitations in application. *Fa Yi Xue Za Zhi*. 2009;**25(5)**:380–382.

28. De Leo D, Evans R. *International suicide rates and prevention strategies*. Cambridge (MA): Hogrefe & Huber; 2004.

29. Manoranjitham SD, Rajkumar AP, Thangadurai P, Prasad J, Jayakaran R, Jacob KS. Risk factors for suicide in rural south India. *Br J Psychiatry*. 2010;**196(1)**:26–30.

30. Zhang J, Xiao S, Zhou L. Mental disorders and suicide among young rural Chinese: A case-control psychological autopsy study. *Am J Psychiatry*. 2010;**167(7)**:773–781.

31. Li XY, Phillips MR, Zhang YP, Xu D, Yang GH. Risk factors for suicide in China's youth: A case-control study. *Psychol Med*. 2008;**38(3)**:397–406.

32. Kim MH, Jung-Choi K, Jun HJ, Kawachi I. Socioeconomic inequalities in suicidal ideation parasuicides and completed suicides in South Korea. *Soc Sci Med*. 2010;**70(8)**:1254–1261.

33. Pridmore S, McArthur M. Suicide and reputation damage. *Australas Psychiatry*. 2008;**16(5)**:312–316.

34. Pridmore S. *Suicide and predicament: Life is a predicament* [Internet]. Bentham Publishers; 2010 [cited 2011 MMM DD]. Available from: http://www.bentham.org/ebooks/9781608051694/index.htm.

35. Cavanagh JT, Owens DG, Johnstone EC. Life events in suicide and undetermined death in south-east Scotland: A case-control study using the method of psychological autopsy. *Soc Psychiatry Psychiatr Epidemiol*. 1999;**34(12)**:645–650.

36. Shiner M, Scourfield J, Fincham B, Langer S. When things fall apart: Gender and suicide across the life-course. *Soc Sci Med*. 2009;**69(5)**:738–746.

37. Foster T. Adverse life events proximal to adult suicide: A synthesis of findings from psychological autopsy studies. *Arch Suicide Res*. 2011;**15(1)**:1–15.

38. Pouliot L, De Leo D. Critical issues in psychological autopsy studies. *Suicide Life Threat Behav*. 2006;**36(5)** :491–510.

39. Summerfield D. Cross-cultural perspectives on the medicalization of human suffering. In: Rosen GM, editor. *Posttraumatic stress disorder. Issues and controversies*. Chichester (UK): John Wiley & Sons Ltd; 2004. p. 233–245.

40. Jacob KS. The prevention of suicide in India and the developing world: The need for population-based strategies. *Crisis*. 2008;**29(2)**:102–106.

41. Jacob KS. The cultures of depression. *Natl Med J India*. 2006;**19(4)**:218–220.

42. Stuart vs Kirkland-Veenstra [2009] HCA 15 (High Court of Australia). In: McDonald B, Anderson R, Yeo S. Case supplement for cases on Torts: 4th edition. Sydney (AU): The Federation Press; 2009.

Case Report

Retrocaval Ureter: The Importance of Intravenous Urography

Radhiana Hassan, Azian Abd Aziz, Siti Kamariah Che Mohamed

Submitted: 21 Dec 2010
Accepted: 30 Jan 2011

Department of Radiology, Kulliyyah of Medicine, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

Abstract

Retrocaval ureter is a rare cause of hydronephrosis. Its rarity and non-specific presentation pose a challenge to surgeons and radiologists in making the correct diagnosis. Differentiation from other causes of urinary tract obstruction, especially the more common urolithiasis, is important for successful surgical management. Current practice has seen multislice computed tomography (MSCT) rapidly replaces intravenous urography (IVU) in the assessment of patients with hydronephrosis due to suspected urolithiasis, especially ureterolithiasis. However, MSCT, without adequate opacification of the entire ureter, may allow the physician to overlook a retrocaval ureter as the cause of hydronephrosis. High-resolution IVU images can demonstrate the typical appearance that leads to the accurate diagnosis of a retrocaval ureter. We reported a case that illustrates this scenario and highlights the importance of IVU in the assessment of a complex congenital disorder involving the urinary tract.

Keywords: *computed tomography, hydronephrosis, ureteral diseases, urography, urology*

Introduction

Retrocaval ureter is a rare condition that results from an anomaly in the development of the inferior vena cava (1). The incidence was reported to be approximately 1 in 1000 people, with male predominance (2). The anomalous vessel compresses the ureter, causing varying degrees of hydronephrosis. The patients are usually 30 to 40 years of age at the time of diagnosis due to the gradual development of hydronephrosis. Imaging studies are usually sufficient for an accurate pre-operative diagnosis, which is important for successful surgical intervention (2).

Case Report

A 62-year-old man was referred to the urology clinic due to incomplete voiding and dribbling of urine for the past 5 years. Clinical examination was unremarkable except for a mildly enlarged prostate gland. Laboratory investigations were normal. Ultrasound (US) of the abdomen and pelvis showed a mildly enlarged prostate. The patient was diagnosed and treated for benign prostatic hypertrophy. During the US examination, right hydronephrosis and proximal hydroureter were incidentally discovered. There was no calculus detected. Because there was evidence of a right obstructed system, abdomen and pelvis multislice computed tomography (MSCT) was performed to rule out right ureteric calculus, which could be missed on US. MSCT showed a persistent right hydronephrosis and hydroureter but did not demonstrate any calculus. The right ureter was dilated up to its midlevel, but not traceable along the expected course distally, mainly due to poor filling of contrast within the ureter. Correlating with US findings, there is a possibility of a right ureteric stricture from a previous passage of calculus. Based on US and CT findings, cystoscopy was then performed, and kinking of the right ureter at level L3 was noted. The right ureter proximal to the kink was dilated with no intraluminal lesion seen. A right ureteric stent was then inserted. This patient had an intravenous urography (IVU) done after the procedure, and the findings were characteristic of a retrocaval ureter (Figure 1). The right retrocaval ureter could actually be seen when the axial CT images were retrospectively reviewed (Figure 2), but this was not demonstrated on the multiplanar reformatted images or the 3-dimensional reconstructed images because of poor contrast opacification of the distal ureter (Figure 3). A retrograde pyelogram (RPG) performed 2 months later showed no ureteric calculus. The patient recovered well after the removal of the ureteric stent, but he refused further surgical intervention.

Discussion

Changing practice patterns have led to MSCT replacing IVU in the assessment of patients with suspected urolithiasis, especially ureteric calculus (3,4). MSCT is preferred over IVU by physicians because of its high sensitivity (96%), specificity (99%), and accuracy (96%) for the detection of ureteric calculus (5). MSCT is fast, widely available, can be done with or without contrast, depending on clinical indication, and can also show signs of urinary tract obstruction. However, in complex cases of congenital anomaly, the diagnosis may be missed due to its rarity and subtle nature. As illustrated in our patient, MSCT scan was not able to visualise the entire right ureter during the excretory phase due to pooling of contrast in the dilated renal pelvis and proximal ureter. Thus, the multiplanar reformatted and 3-dimensional reconstruction image was not useful in this instance. A normal size non-opacified ureter can be difficult to trace; therefore, a congenital anomaly, such as a retrocaval ureter, can be missed if it is not considered. One study reported low sensitivity (59%) in the detection of ureteral abnormality (ureteral duplication) on axial non-contrasted CT, even when the images were reviewed by radiologists who specialised in genitourinary imaging (6).

IVU has some advantages; it can provide good image resolution and the examination can be modified according to the clinical needs, for example, obtaining delayed images or changing the patient's position to try to visualise the entire length of the ureter. Although not diagnostic, the appearance of retrocaval ureter on IVU is typical and is highly suggestive of the diagnosis (7). MSCT, however, is performed to confirm the diagnosis and to rule out other causes of ureteral deviation. On a CT scan, the lateral placement

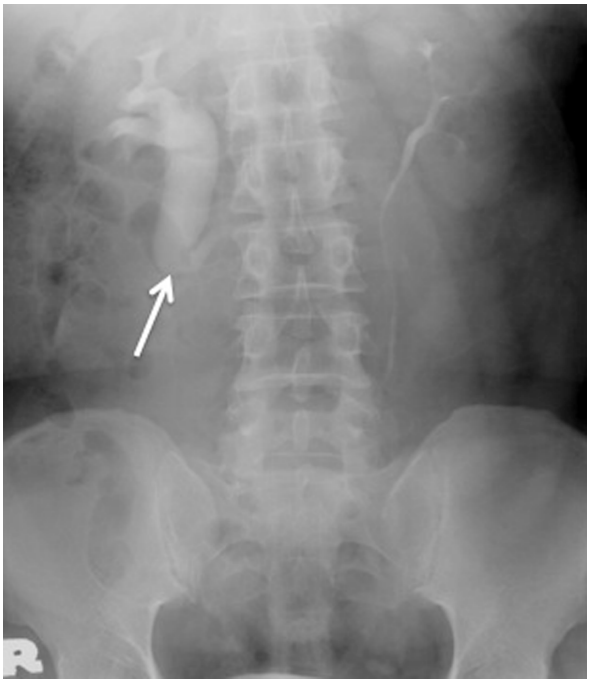


Figure 1: Intravenous urogram showing right-sided hydronephrosis and the dilation of the proximal ureter up to the level of the L3 transverse process. The medial deviation of the ureter at this level (arrow) gives rise to the typical fish hook or reversed S appearance.

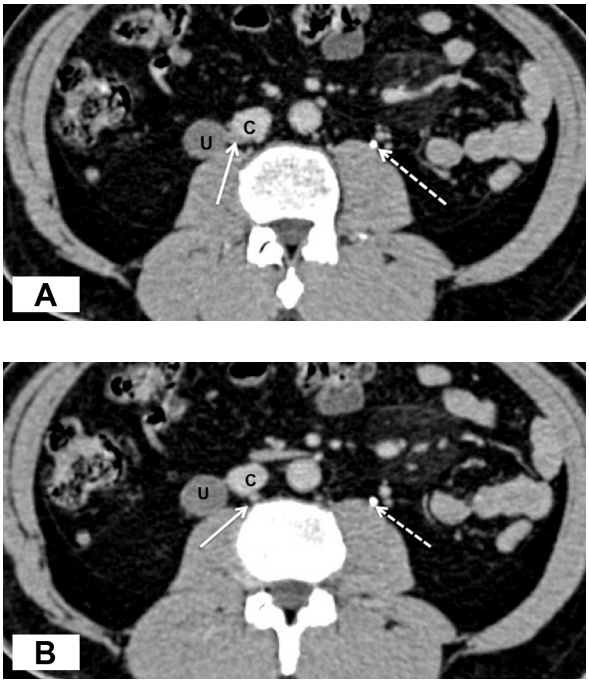


Figure 2: Contrast-enhanced computed tomography scan in the axial view showing (A) the dilated right ureter (U) proximal to its obstruction. The ureter follows a medial course at this level (solid arrow), posterior to the inferior vena cava (C). At a lower scan (B), the retrocaval location of the right ureter is medial compared with the normal location of the left ureter (dashed arrow).

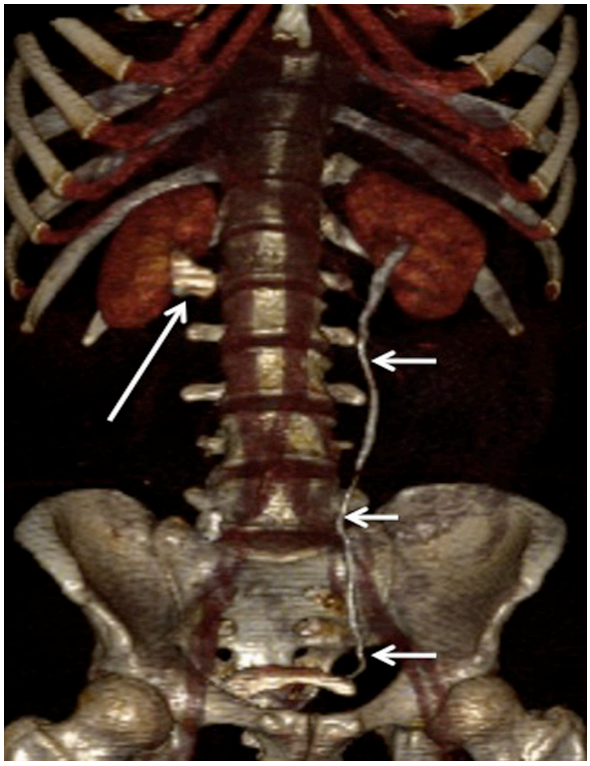


Figure 3: A 3-dimensional reconstruction image from the excretory phase of computed tomography showing the normal course and calibre of the left ureter (short arrows). However, the right ureter was not visualised due to inadequate opacification and contrast filling. The contrast is seen pooling in the dilated right renal pelvis and proximal ureter (long arrow).

of the IVC to the right pedicle is found in all patients with retrocaval ureters but in only 6% of normal patients (1,8). Recently, MRI was also reported to be useful in demonstrating retrocaval ureter and correlated well with IVU (2,9). It has the advantage of being radiation free and of providing multiplanar images. However, it may not be practical in our setting due to its high cost and limited availability in some health centres. In our case, the main focus was to detect possible ureteric calculus that was thought to cause the obstruction; therefore, when US and CT did not show any calculus, invasive procedures, such as cystoscopy and RPG, could be performed.

The radiological features of retrocaval ureter on IVU are divided into 2 types. In Type 1, the ureter crosses behind the IVC at the level of the 3rd lumbar vertebra and has a fish hook–shaped or S-shaped deformity of the ureter. It is also known as the low loop retrocaval ureter. Marked hydronephrosis is seen in over 50% of patients. In Type 2, the retrocaval segment is at the same level as the renal pelvis; the sickle-shape appearance of the involved ureter can be resolved on IVU. Type 2 generally causes mild hydronephrosis and is less common compared with Type 1 (10).

Treatment depends on the clinical presentation, the severity of hydronephrosis, and the impairment of renal function. Patients with mild hydronephrosis without renal impairment or any associated complication can be managed conservatively with periodic examinations (2). Ureteroureteral reanastomosis anterior to the IVC with resection of the retrocaval segment is the favoured surgical treatment, with good results reported (2).

IVU, which is an old and traditional examination that is considered to be almost obsolete by some, is still valuable for the assessment of genitourinary tract pathology, especially congenital anomaly, as demonstrated in this case.

Authors' Contribution

Conception and design, drafting of the article: RH
Collection, assembly, analysis and interpretation of the data, critical revision and final approval of the article: RH, AAA, SKCM

Correspondence

Dr Radhiana Hassan
MD (USM), MMed Radiology (USM)
Department of Radiology, Kulliyyah of Medicine
International Islamic University Malaysia
Bandar Indera Mahkota
25200 Kuantan
Pahang, Malaysia
Tel: +609-557 2056
Fax: +609-514 9396
Email: radhianahassan@gmail.com

References

1. Lautin EM, Haramati N, Frager D, Friedman AC, Gold K, Kurtz A, et al. CT diagnosis of circumcaval ureter. *AJR Am J Roentgenol.* 1988;**150(3)**:591–594.

2. Uthappa MC, Anthony D, Allen C. Case report: Retrocaval ureter: MR appearances. *Br J Radiol.* 2002;**75(890)**:177–179.

3. Chen MY, Zagoria RJ, Saunders HS, Dyer RB. Trends in the use of unenhanced helical CT for acute urinary colic. *AJR Am J Roentgenol.* 1999;**173(6)**: 1447–1450.

4. Kambadakone AR, Eisner BH, Catalano OA, Sahani DV. New and evolving concepts in the imaging and management of urolithiasis: Urologists' perspective. *Radiographics*. 2010;**30(3)**:603–623.

5. Novelline RA, Rhea JT, Rao PM, Stuk JL. Helical CT in emergency radiology. *Radiology*. 1999;**213(2)**: 321–339.

6. Eisner BH, Shaikh M, Uppot RN, Sahani DV, Dretler SP. Genitourinary imaging with noncontrast computerized tomography–Are we missing duplex ureters? *J Urol*. 2008;**179(4)**:1445–1448.

7. Perimenis P, Gyftopoulos K, Athanasopoulos A, Pastromas V, Barbalias G. Retrocaval ureter and associated abnormalities. *Int Urol Nephrol*. 2002;**33(1)**:19–22.

8. Singh DD, Sanjeev P, Sharma RK. Images: Spiral CT evaluation of circumcaval ureter (retrocaval ureter). *Indian J Radiol Imaging*. 2001;**11(2)**:83–84.

9. Dillon B, Goodman TR. A periureteric venous ring diagnosed by MRI: An unusual cause of flank pain. *Pediatr Radiol*. 2006;**36(6)**:564–565.

10. Bateson EM, Atkinson D. Circumcaval ureter: A new classification. *Clin Radiol*. 1969;**20(2)**:173–177.

Case Report

Kimura's Disease: A Rare Cause of Nephrotic Syndrome with Lymphadenopathy

Siti Kamariah Othman[1], Kamaliah Mohd Daud[1], Nor Hayati Othman[2]

Submitted: 12 Dec 2010
Accepted: 14 Apr 2011

[1] *Department of Medicine, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

[2] *Department of Pathology, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

Abstract

Kimura's disease is a rare condition and typically presents as non-tender subcutaneous swellings in the head and neck region, usually in the pre-auricular and submandibular areas. It is associated with lymphadenopathy (both local and distal), marked peripheral eosinophilia, and an elevated IgE level. It can easily be mistaken for a malignant disorder. Fine needle aspiration can be misleading, and a diagnosis is established only by histopathological examination. Renal involvement, which may affect up to 60% of patients, is the only systemic manifestation. We report a case of Kimura's disease in a Malay patient who was associated with steroid-responsive nephrotic syndrome.

Keywords: *eosinophilic granuloma of soft tissue, Kimura's disease, lymphadenopathy, neck, nephrotic syndrome*

Introduction

Kimura's disease is a benign, chronic inflammatory soft tissue disorder of unknown origin. Although it is rare, most cases of Kimura's disease have originated in China, Japan, or Southeast Asia, and the disease is uncommon in Caucasians and rare in Africans. There is a marked male predominance. The peak age of onset is during the third decade of life.

Kimura's disease was first described in 1937 in the Chinese literature by HT Kimm and C Szeto and initially was recognised as "eosinophilic hyperplastic lymphogranuloma". The definitive histological description was published by Kimura et al. (1) in 1948, and thus, the disease has borne the author's name. Since that time, there has been a gradual increase in the number of reports of the disease.

Case Report

A 40-year-old Malay male first presented in August 2005 with generalised body oedema of 2 weeks' duration. He was previously well, without any history of hospitalisation. He reported multiple painless right neck swellings for the last 30 years, which were gradually increasing in size and were not associated with any local or systemic symptoms. There was no history of chronic cough, prolonged fever, reduced weight, night sweats, or low back pain suggestive of tuberculosis (TB) infection. There was no family history of renal or autoimmune disease.

Examination revealed a normotensive man with bilateral gross leg oedema and ascites. Three cervical lymph nodes were palpable at the posterior triangle of the right neck, the largest being 2 cm in diameter. The lymph nodes were firm, non-matted, non-tender, smooth surfaced, and mobile. There was also a cystic lesion located behind the right ear, measuring 2 × 2 cm, non-tender, and mobile. His lungs were clear. There was no hepatosplenomegaly and no other palpable regional lymph nodes.

Investigations revealed a haemoglobin level of 14.6 g/dL, a white cell count of 10.47×10^9 cells/L with 8.7% eosinophils, and a normal platelet count. Urinalysis revealed 4+ albuminuria and nil for sugar and red blood cells. The patient's 24-hour total urinary protein excretion was 8.11 g, and his serum creatinine level was 149 μmol/L. Electrolyte levels were normal. Albumin and globulin concentrations were 16 g/L and 24 g/L, respectively. The erythrocyte sedimentation rate was 89 mm/hour, and total cholesterol level was 19.6 mmol/L. The Mantoux test was negative. Sputum samples for direct smear were negative. Sputum culture for *Mycobacterium* was still pending at that time.

The tests for hepatitis B surface antigen and anti-hepatitis C virus antibody were negative. The antinuclear factor and rheumatic factor tests were negative, and complement C3 and C4 levels were within normal limits. The antistreptolysin titre was negative. Ultrasound examination showed normal-sized kidneys with increased echogenicity on the right kidney, and no calculus was seen. A chest radiograph did not show any abnormal opacities, cavitations, or perihilar lymph nodes.

A biopsy of the lymph nodes showed haemorrhage and hyperplastic lymphoid follicles with germinal centres. The paracortical areas showed eosinophilic infiltrate and proliferation of post-capillary venules (Figure 1). No evidence of malignancy or metastatic deposits was observed. The findings were consistent with Kimura's disease. Renal biopsy was not performed in this patient because of his refusal.

The patient was started on prednisolone (1 mg/kg/day) and diuretics. Subsequently, his serum creatinine level reduced to 107 µmol/L. He was then discharged on oral prednisolone (80 mg once daily), oral furosemide (40 mg twice daily), and oral atorvastatin (20 mg once daily).

All the enlarged right cervical lymph nodes shrunk after prednisolone treatment was initiated. The sputum culture that was taken during his first admission (August 2005) yielded *Mycobacterium tuberculosis*, which was sensitive to isoniazid, streptomycin, rifampicin, and ethambutol. The patient was started on an anti-TB drug regime that consisted of ethambutol, isoniazide, rifampicin, pyrazinamide (EHRZ) in February 2006. Prednisolone prescription was continued together with the anti-TB drugs. He had a history of relapsed nephrotic syndrome in June 2006 while on a tapering dosage of prednisolone.

From July 2007 until the present, his nephrotic syndrome was in remission. His serum creatinine level was 120 mmol/L (static), total cholesterol level was 4.70 mmol/L, and serum albumin level was 44 g/dL. His cervical lymph nodes were also resolved. He is currently on maintenance prednisolone (5 mg once daily), oral enalapril (5 mg once daily), and oral atorvastatin (20 mg once daily).

Discussion

Our patient was a 40-year-old male of Malay ethnicity who developed nephrotic syndrome 30 years after the onset of lymphadenopathy, which is the longest such duration compared with previous reported cases. His eosinophil count was only at a borderline elevated level (8%) on initial

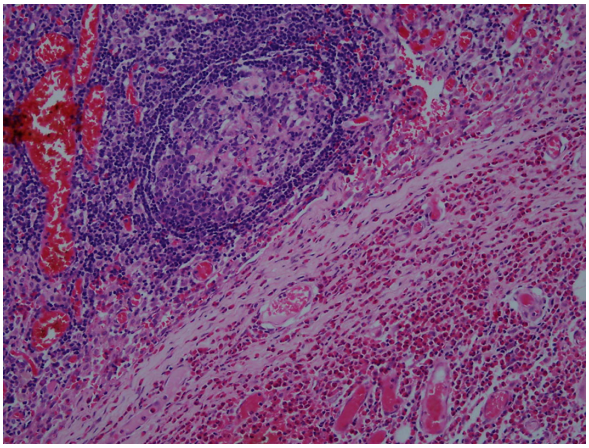


Figure 1: The paracortical areas show eosinophilic infiltration and proliferation of post-capillary venules. No evidence of malignancy or metastatic deposits is seen (200× magnification).

presentation, and peripheral eosinophilia is the most consistent feature in most cases of Kimura's disease.

The differential diagnosis of nephrotic syndrome associated with lymphadenopathy in this patient included lymphoma, especially Hodgkin's lymphoma. Minimal change disease was found in Hodgkin's lymphoma associated with nephrotic syndrome. However, the lymph node biopsy was not suggestive of lymphoma in this case, and the patient did not have constitutional symptoms consistent with lymphoma. Membranous nephropathy has also been associated with TB. Even though the patient's sputum smear was subsequently positive for TB, the initial lymph node biopsy did not suggest TB lymphadenitis. Acute or chronic lymphocytic leukemias can also be associated with nephrotic syndrome, typically minimal change disease, but both were unlikely in this patient because he had a normal blood count and the lymphadenopathy was longstanding and confined only to the neck region.

Until 1982, 21 cases of nephropathy in patients with Kimura's disease had been reported in the literature, and all involved male patients (2). A subsequent review covering the period from 1981 to 1998 revealed an additional 12 cases, 10 of which involved male patients (3). A search by Yuen et al. (5) of the English medical literature from 1998 to 2004 revealed 8 more cases of Kimura's disease with nephropathy. The patients were 9 to 35 years of age and were males.

The renal manifestations of Kimura's disease are as membranous glomerulonephritis, minimal change glomerulonephritis, diffuse proliferative glomerulonephritis, mesangial proliferative glomerulonephritis, and nephritic syndrome (4,6). In our patient, the type of renal involvement could not be ascertained because he refused renal biopsy. It is intuitive to speculate that male gender is a risk factor for renal involvement in Kimura's disease, but a conclusion cannot yet be reached given the small number of reported cases (5).

Management strategies range from conservative observation for asymptomatic patients to surgical excision, steroid therapy, or radiotherapy for symptomatic patients (4), but no method has been proven to be the best, and recurrence is common. Steroids, for example, are effective in inducing remission of subcutaneous masses and lymphadenopathy in Kimura's disease, but relapse may occur when treatment is stopped (7). Several reports describing a steroid-responsive nephrotic syndrome have shown a reduction in the size of subcutaneous nodules with steroids, implying a common aetiopathogenesis of the renal lesion and Kimura's disease. The nephrotic syndrome in our patient was steroid responsive, and his enlarged lymph nodes also resolved after steroid therapy. His nephrotic syndrome, however, relapsed while on a tapering dose of prednisolone, and he had to be maintained on a low-dose steroid. Radiotherapy could be a highly effective alternative when other treatment modalities are unsuccessful (8). In summary, Kimura's disease should be considered as a differential diagnosis in male Asian patients with proteinuria or nephrotic syndrome associated with lymphadenopathy.

Optimal treatment regimens and long-term prognosis for renal lesions in patients with Kimura's disease are largely unknown due to the rarity of these lesions and the lack of long-term follow-up data in the literature. There is no evidence that nephropathy in Kimura's disease carries an exceptionally poor outcome.

Authors' Contributions

Analysis and interpretation of data: NHO
Drafting of the article: SKO
Critical revision of the article: KMD

Correspondence

Dr Siti Kamariah Othman
MD (USM)
Department of Medicine
Level 7, Hospital Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan, Malaysia
Tel: +6013-709 6579
Email: dr_pegaga@yahoo.com.my

References

1. Kimura T, Yoshimura S, Ishikawa E. On the unusual granulation combined with hyperplastic changes of lymphatic tissue. *Trans Soc Pathol.* 1948;**37**: 179–180.

2. Yamada A, Mitsuhashi K, Miyakawa Y, Kosaka K, Takehara K, Iijima M, et al. Membranous glomerulonephritis associated with eosinophilic lymphofolliculosis of the skin (Kimura's disease): Report of a case and review of the literature. *Clin Nephrol.* 1982;**18(4)**:211–215.

3. Natov SN, Strom JA, Ucci A. Relapsing nephrotic syndrome in a patient with Kimura's disease and IgA glomerulonephritis. *Nephrol Dial Transplant.* 1998;**13(9)**:2358–2363.

4. Messina-Doucet MT, Armstrong WB, Allison G, Pena F, Kim JKV. Kimura's disease: Two case reports and a literature review. *Ann Otol Rhinol Laryngol.* 1998;**107(12)**:1066–1071.

5. Yuen SK, Yong SP, Tsui WMS, Siu FY, Tsui HS, Cheung KO. Minimal change nephrotic syndrome with IgM deposits in Kimura's disease: A case report and literature review. *Hong Kong J Nephrol.* 2004;**6(2)**:97–102.

6. Hui PK, Chan JK, Ng CS, Kung IT, Gwi E. Lymphadenopathy of Kimura's disease. *Am J Surg Pathol.* 1989;**13(3)**:177–186.

7. Nakahara C, Wada T, Kusakari J, Kanemoto K, Kinugasa H, Sibasaki M, et al. Steroid-sensitive nephrotic syndrome associated with Kimura disease. *Pediatr Nephrol.* 2000;**14(6)**:482–485.

8. Kilciksiz S, Calli C, Eski E, Topcugil F, Bener S. Radiotherapy for Kimura's disease: Case report and review of the literature. *J BUON.* 2007;**12(2)**: 277–280.

Case Report

Y-Stent–Assisted Coil Embolisation of Wide-Necked Aneurysms Using a New Fully Retrievable and Detachable Intracranial Stent: Report of Two Cases

Ahmad Sobri Muda[1], Ahmad Razali Md Ralib[2], Yazmin Yaacob[1], Rozman Zakaria[1], Azizi Abu Bakar[3]

Submitted: 30 Jan 2011
Accepted: 26 Apr 2011

[1] *Department of Radiology, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur, Malaysia*

[2] *Department of Radiology, Kulliyyah of Medicine, International Islamic University Malaysia, 25710 Kuantan, Pahang, Malaysia*

[3] *Department of Surgery, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur, Malaysia*

Abstract

Endovascular treatment of wide-necked aneurysms poses a challenge for the endovascular therapist. The Y-stent–assisted technique has been used for stent-assisted coil embolisation for wide-necked bifurcation aneurysms. This technique has been described for basilar tip aneurysms and middle cerebral artery bifurcation aneurysms using Neuroform and Enterprise stents. We report 2 cases of wide-necked bifurcation aneurysms that were treated with Y-stent–assisted coil embolisation using a new, fully retrievable and detachable intracranial stent (Solitaire AB™). We describe the advantages of a fully retrievable and detachable stent and its feasibility of forming a Y configuration.

Keywords: *endovascular techniques, intracranial aneurysm, neurosurgery, stents, therapeutic embolisation*

Introduction

Endovascular treatment (EVT) is an established treatment in most patients with ruptured and unruptured aneurysms (1,2). However, wide-necked aneurysms present a challenge for the endovascular therapist. Many techniques assist in the embolisation of these aneurysms, such as balloon remodelling (3), jailed microcatheter (4), horizontal stent–assisted (5), and Y-stent–assisted (6). Stent-assisted techniques have the most advantages over balloon remodelling techniques (7). Many types of stents are available, from open-cell to closed-cell designs, each with its own advantages and disadvantages. The Solitaire AB™ stent (ev3 Inc., Irvine, CA) is a new stent for intracranial stenting that is fully retrievable and detachable, features that are not available in other types of stents (7,8). We present our experience with Y-stent–assisted coil embolisation of wide-necked bifurcation aneurysms using Solitaire AB™ stents. To the best of our knowledge, there have been only 2 previous cases in the English medical literature in using Y-stent–assisted coil embolisation of wide-necked bifurcation aneurysms using Solitaire AB™ stents (8).

Case Series

Case 1

A 68-year-old woman presented with a sudden loss of consciousness. On arrival, she had a Glasgow Coma Scale of 6/15. Unenhanced computed tomography (CT) scan of the brain revealed extensive subarachnoid haemorrhages, and the subsequent CT angiography showed a large wide-necked basilar tip aneurysm that measured 5.8 × 6.3 mm with a neck that measured 4.3 mm (Figure 1). After an interdisciplinary discussion and an explanation of risks, benefits, and alternatives, we proceeded with EVT using the Y-stent–assisted coil embolisation technique. Due to the tortuosity and atherosclerotic posterior circulation, especially of the posterior cerebral arteries, we postulated that we would have difficulties in good stent placement on a single release. Therefore, we used the new Solitaire AB™ stent, which is fully retrievable if the stent placement is not optimal.

The procedure was performed under general anaesthesia. Because the patient was on long-term dual antiplatelet therapy (clopidogrel and aspirin) for ischaemic heart disease, a loading dose of clopidogrel was not needed. Bilateral common femoral artery punctures were performed. A 6F guiding catheter equipped with a continuous heparinised flushing system was advanced via right femoral artery puncture with the tip at the proximal left vertebral artery. Working projections were identified and baseline runs were performed. A microcatheter (Prowler 0.021", Cordis, Miami Lakes, FL) with a GT double curve microwire (Terumo, Tokyo, JP) was advanced into the left P1 segment with the tip placed at the distal left P1 segment. A fully retrievable and detachable stent (Solitaire AB™, 4 × 20 mm) was delivered and fully deployed but not detached. On the check run, we noted a significant flow reduction into the left posterior cerebral artery. We fully retrieved and repositioned the stent without causing a significant flow reduction, and the stent was detached.

Subsequently, we navigated the microcatheter through the stent strut into the right P1 segment. A second stent was deployed and detached through the first stent mesh to form a Y configuration (Figures 2a and 2b). Loose coiling of the basilar tip aneurysm was performed using 2 detachable coils (Figure 2c). Post-procedural angiographic runs showed no immediate compromise to the major supply of the posterior circulation with occlusion and a significant slowing of flow within the basilar tip aneurysm (Figure 2d). The patient was discharged after 3 weeks in the intensive care unit and 2 weeks in the general ward and was ambulated with assistance.

The patient returned for a follow-up visit about 4 months post-embolisation. During the follow-up visit, she walked with assistance. Unfortunately, she fell and suffered a loss of consciousness during a shopping trip after the follow-up visit. An urgent CT scan of the brain showed a massive right temporoparietal subdural haemorrhage with mass effect and internal herniation. The patient underwent an urgent craniotomy due to the massive subdural haemorrhage. Unfortunately, the patient did not recover from the surgery and died about 3 days post-operatively.

Case 2

A 54-year-old gentleman presented with frequent headache. A CT scan of the brain followed by a CT angiogram revealed a large wide-necked left middle cerebral artery (MCA) aneurysm that measured 11.1 × 11.2 mm with a neck that measured 5.2 mm. There was also another aneurysm in the left posterior communicating artery. There was no evidence of intracranial haemorrhage. The patient was keen for treatment and decided on an EVT. Initially, the patient underwent an embolisation of the left posterior communicating artery aneurysm. We then planned for a Y-stent–assisted coil embolisation of the large left MCA aneurysm at a later date.

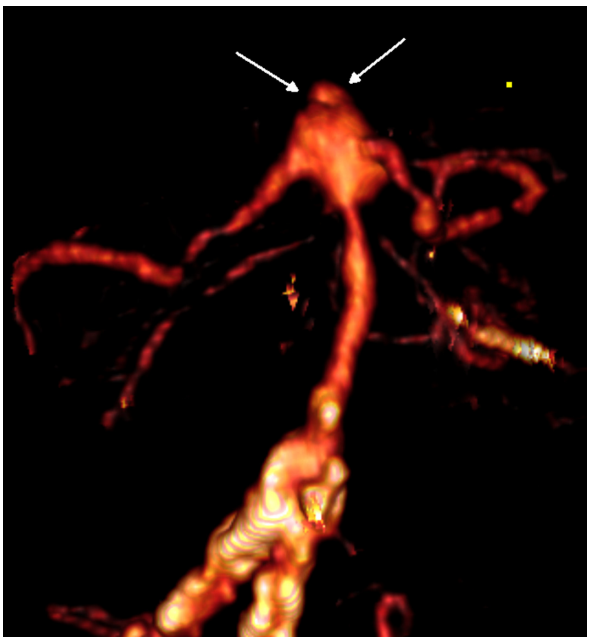


Figure 1: Three-dimensional reconstruction of computed tomography angiography showing a wide-necked basilar tip aneurysm (white arrows) measuring 5.8 × 6.3 mm with a neck measuring 4.3 mm.

Clopidogrel (75 mg daily) and aspirin (150 mg daily) were started 5 days prior to the procedure. EVT was performed under general anaesthesia. Bilateral common femoral artery punctures were done. A 6F sheath was used with the tip of the guiding catheter in the proximal left internal carotid artery. We used the Solitaire AB™ stent with the Leo stent (Balt, Montmorency, FR) to form a Y configuration and loosely coil the aneurysm. A microcatheter (Prowler 0.021") was advanced into the M2 segment of the left MCA with the tip beyond the aneurysm neck. The Solitaire AB™ stent was delivered and fully deployed but not detached (Figure 3a). The microcatheter (Prowler 0.021") was pushed through the Solitaire AB™ stent struts without difficulty with the tip within the other M2

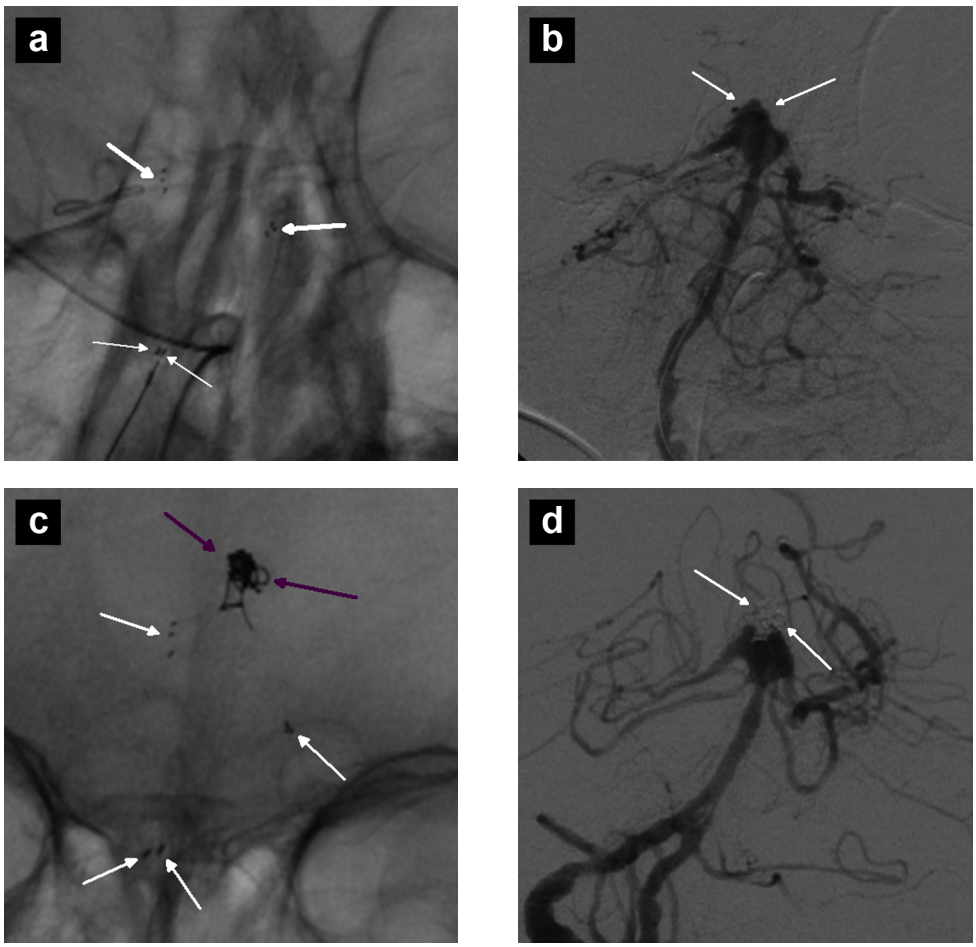


Figure 2: (a) Post-deployment of both Solitaire AB™ stents to form the Y configuration. The proximal markers (thin white arrows) and distal markers (thick white arrows) of the Solitaire AB™ stent are visualised. (b) Angiographic run post-deployment of Solitaire AB™ stents with no compromise of flow to the posterior circulation. The basilar tip aneurysm is visualised (white arrows). (c) Post-coiling of the basilar tip aneurysm (black arrows). The proximal and distal markers of the Solitaire AB™ stent are visualised (white arrows). (d) Post-embolisation angiographic run showing about a 50% obliteration of the basilar tip aneurysm (white arrows) with no compromise of flow to the posterior circulation.

branch. A Leo stent (2.5 × 18 mm) was delivered (Figure 3a). A check run showed both stents formed a proper Y configuration with patency of both M2 branches (Figure 3b). The Solitaire AB™ stent was detached. The aneurysm was loosely packed with 2 detachable coils that were deployed via an Excelsior SL 10 microcatheter (Boston Scientific, Fremont, CA) cannulated through the Solitaire AB™ stent struts (Figure 3c).

Post-embolisation angiographic runs showed a slowing of contrast filling into the aneurysm (Figure 3d). The patient did well post-procedure and was discharged 4 days later.

On a follow-up angiogram 3 months later, only about 30% of the MCA aneurysm remained opacified (Figure 4) with patency of both M2 branches. The patient was stable with no complaints, and clopidogrel and aspirin prescription were continued.

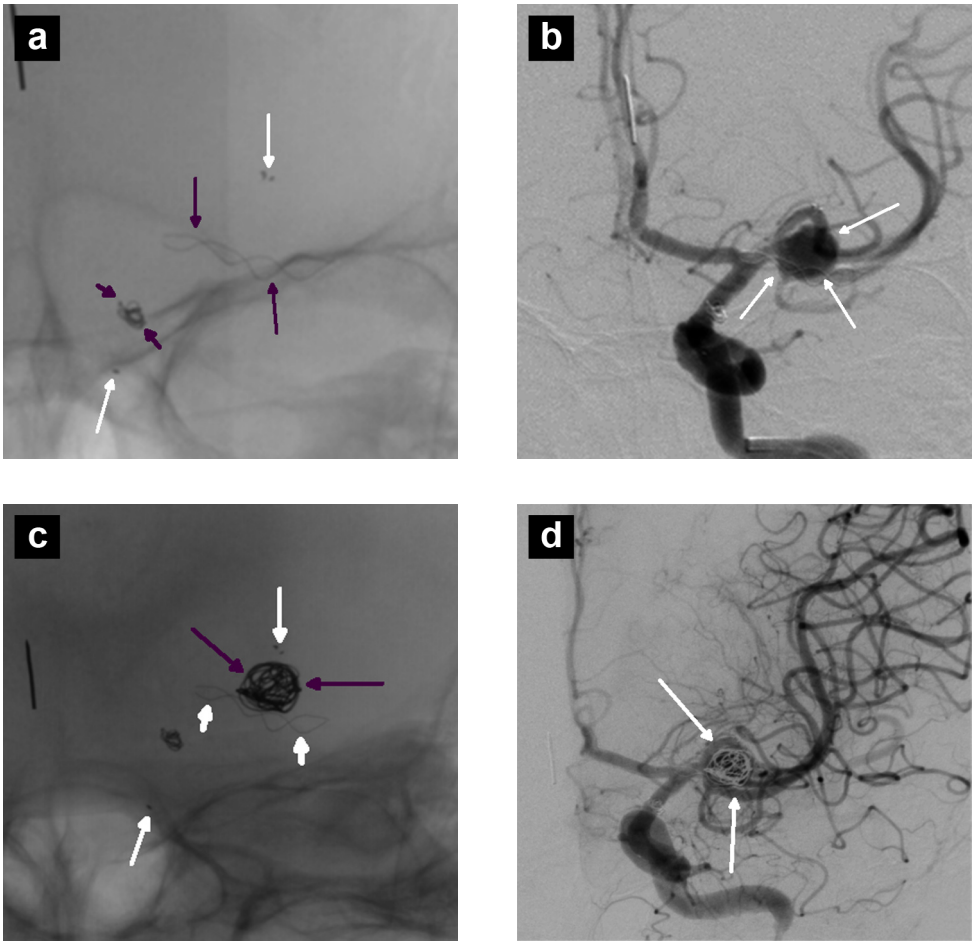


Figure 3: (a) Post-deployment of Solitaire AB™ (white arrows) and Leo (long black arrows) stents to form the Y configuration. The coils within the posterior communicating artery aneurysm are also visualised (short black arrows). (b) Angiographic run post-deployment of Solitaire AB™ and Leo stents with patency of both M2 branches. The MCA aneurysm is visualised (white arrows). (c) Post-coiling of the MCA aneurysm (black arrows). The Solitaire AB™ (long white arrows) and Leo (short white arrows) stents are visualised. (d) Post-embolisation angiographic run showing about a 50% obliteration of the MCA aneurysm (white arrows) with patency of both M2 branches.

Discussion

There are various methods for parent artery reconstruction for wide-necked aneurysms prior to coil embolisation: these include balloon remodelling, jailed microcatheter, horizontal stent–assisted, and Y-stent–assisted. Jailed microcatheter is a technique in which a microcatheter is first placed into the aneurysm and "jailed" after the stent deployment, which helps stabilise the device during the deployment of coils (4). However, this method does not allow for microcatheter repositioning after the stent has been deployed.

The balloon remodelling technique or balloon-assisted coil embolisation is a technique in which the balloon is inflated within the parent artery to achieve a dense packing of the aneurysm (3). However, it is associated with a higher percentage of procedure-related complications compared with conventional coil embolisation (3).

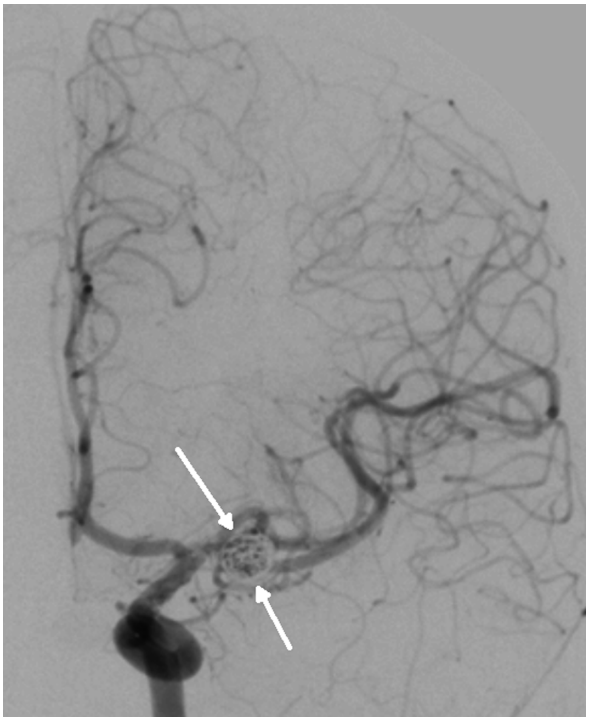


Figure 4: Left internal carotid angiogram 3 months post-embolisation showing only about 30% of the MCA aneurysm opacified with patency of both M2 branches.

Stents have revolutionised the treatment of wide-necked aneurysms. Stents provide structural support for coil embolisation, prevent coil herniation, allow for an increased packing density, and have an impact on flow diversion (4,9). The placement of a stent across the neck of the aneurysm alters the blood flow pattern and disrupts the flow into the aneurysm, thus allowing for a spontaneous thrombosis of the aneurysm (10).

Wide-necked aneurysms arising from vessel bifurcation, such as basilar tip aneurysm and middle cerebral artery bifurcation aneurysm, provide a bigger challenge for the endovascular therapist. Two treatment methods have been described for these wide-necked bifurcation aneurysms: horizontal stent–assisted and Y-stent–assisted.

The horizontal stent–assisted technique has been described as an alternative to the Y-stent–assisted technique. This technique is defined as the horizontal deployment of a stent across the neck of a terminal aneurysm, which is achieved by the navigation of the stent through the circle of Willis (5). Basilar tip aneurysms are accessed from the carotid system via the posterior communicating artery, and carotid tip aneurysms are accessed from the contralateral carotid artery via the anterior communicating artery. The advantage of this technique over the Y-stent–assisted technique is a reduction in the number of stent requirements, which reduces cost and the risk of thrombosis. However, the success of this technique depends on the availability of good-sized anterior or posterior communicating arteries. Siddiqui et al. (5) described 8 aneurysms that were treated successfully with an Enterprise stent (Cordis Neurovascular) using the horizontal stent–assited technique: 6 cases of basilar tip aneurysms and 2 cases of carotid bifurcation aneurysms.

The Y-stent–assisted technique is the other alternative for the treatment of wide-necked bifurcation aneurysms. Lozen et al. (6) described 5 basilar tip aneurysms and 1 middle cerebral artery aneurysm that were treated with Y configuration Neuroform stents (Boston Scientific, Fremont, CA). The Y configuration was successfully established in all 6 patients. For 1 patient, there was some difficulty in the delivery of the second stent due to the presence of friction within the system. The authors postulated that the use of this technique in larger calibre arteries would limit the intraprocedural friction. Rohde et al. (9) described a Y-stent–assisted coil embolisation of a wide-necked anterior communicating artery aneurysm using Enterprise stents. However, because the Enterprise stent has a closed-cell design, there was an initial difficulty in the pushing of the microcatheter through the stent struts, which probably occurred because the tip of the microcatheter was hooked on the cells of the stent. A second stent was also inserted about 4 months later to allow for a "healing-in" of the first stent and to reduce the risk of stent displacement during the insertion of the second stent.

Klish et al. (8) described 2 patients with broad-necked basilar tip aneurysms that were treated using the creation of a stent in a Y configuration at the basilar artery apex using Solitaire AB™ stents. Although Solitaire AB™ stent has a closed-cell design similar to the Enterprise stent, the Solitaire AB™ cell is approximately twice as wide as that of the Enterprise. The Solitaire AB™ allows a 3-mm diameter circle to pass through it. If a 4-mm stent is inserted through the interstices of the stent, it will open to a 3-mm diameter while passing through the interstices of the first stent. This opening is not seen in other commercially available stents. Therefore, Solitaire AB™ provides the best of the open- and closed-cell design features, especially

for Y-stent–assisted coil embolisation. In their series, there were no problems in the guiding of the microcatheter through the struts of the first stent into the other posterior cerebral artery.

In both of our patients, no difficulties in the navigation of the microcatheter through the Solitaire AB™ stent strut or the deployment of the second stent through the first stent struts occurred. Because the Solitaire AB™ cell area allows a 3-mm diameter circle to pass through it, the second stent of the Y configuration need not be the Solitaire AB™ stent. In the second patient with middle cerebral artery bifurcation aneurysm, the Leo stent was used as the second stent for the Y configuration with similar ease.

During the early use of stents for aneurysm treatment, a stent was utilised to provide structural support, prevent coil herniation, and allow for the dense packing of the aneurysms (10). However, in the last few years, it has been noted that a stent alone induces flow reduction within the aneurysm to cause thrombus formation and vessel wall remodelling (11). From an in vitro study of the haemodynamic alteration of a side-wall aneurysm post-stenting using laser Doppler anemometry, a dedicated flow diverter, such as the Silk stent and Phenox flow diverter, was shown to reduce the flow in the inflow zone, outflow zone, and the central dome up to 90% (11). Early studies using the Pipeline embolisation device, which was the first endovascular construct that was specifically engineered to function as a standalone device for the endovascular reconstruction of a segmentally diseased parent vessel, demonstrated a complete angiographic occlusion of cerebral aneurysms in 93% of patients after a 12-month follow-up period (12). In view of this current data, we loosely packed the aneurysm in the second patient in the hopes of a flow diversion effect of the Solitaire AB™ stent to induce thrombosis of the aneurysm.

It was very unfortunate that the first patient died due to an acute subdural haemorrhage as a complication of antiplatelet therapy. The decision to use antiplatelet therapy to reduce thromboembolic complications in elective coil embolisation cases is highly controversial. However, Yamada et al. (13) has shown in a retrospective study that there was a significantly lower symptomatic thromboembolic complication rate in patients who receive antiplatelet therapy compared with those who do not. Although there is higher risk of haemorrhage in patients who receive antiplatelet therapy, the benefits appear to outweigh the risks (13).

Solitaire AB™ is the first fully retrievable and detachable intracranial stent (7,8). A fully retrievable stent allows for the repositioning of the stents in cases of flow compromise. In our patient with a wide-necked basilar tip aneurysm after the first Solitaire AB™ stent was fully delivered and fully deployed, there was a significant flow reduction in the left posterior cerebral artery. Due to this flow reduction, the stent was fully retrieved and repositioned. The stent was only fully detached when we were satisfied that the stent position produced no evidence of flow reduction. This repositioning is one of the advantages of the Solitaire AB™ stent and is not available in other intracranial stents.

Conclusion

Treatment of wide-necked bifurcation aneurysm using the Y-stent–assisted coil embolisation technique is safe and effective. The use of Solitaire AB™ stents in the formation of the Y configuration in this technique is relatively easy, with minimal complications, due to its large cell strut. The Solitaire AB™ stent feature of a fully retrievable stent allows for the repositioning of a fully deployed stent, which is not seen in other types of intracranial stents.

Authors' Contributions

Conception and design: ASM, YY
Provision of patients: YY, RZ, AAB
Collection and assembly of the data: RZ, AAB
Analysis and interpretation of the data, drafting of the article: ARMR, YY
Critical revision of the article: ASM, ARMR, RZ, AAB
Final approval of the article: ASM, ARMR

Correspondence

Dr Ahmad Razali Md Ralib
MBBS (UM), MMed Radiology (USM)
Department of Radiology
Kulliyyah of Medicine
International Islamic University Malaysia
25710 Kuantan
Pahang, Malaysia
Tel: +609-571 6400
Fax: +609-517 8548
Email: arazali@iium.edu.my

References

1. Roy D, Milot G, Raymond J. Endovascular treatment of unruptured aneurysms. *Stroke*. 2001;**32(9)**: 1998–2004.

2. Molyneux AJ, Kerr RS, Yu LM, Clarke M, Sneade M, Yarnold JA, et al. International subarachnoid aneurysm trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: A randomised comparison of effects on survival, dependency, seizures, rebleeding, subgroups, and aneurysm occlusion. *Lancet*. 2005;**366(9488)**:809–817.

3. Sluzewski M, van Rooij WJ, Beute GN, Nijssen PC. Balloon-assisted coil embolization of intracranial aneurysms: Incidence, complications, and angiography results. *J Neurosurg*. 2006;**105(3)**: 396–399.

4. Wanke I, Forsting M. Stents for intracranial wide-necked aneurysms: More than mechanical protection. *Neuroradiology*. 2008;**50(12)**:991–998.

5. Siddiqui MA, Bhattacharya J, Lindsay KW, Jenkins S. Horizontal stent-assisted coil embolisation of wide-necked intracranial aneurysms with the Enterprise stent—A case series with early angiographic follow-up. *Neuroradiology*. 2009;**51(6)**:411–418.

6. Lozen A, Manjila S, Rhiew R, Fessler R. Y-stent-assisted coil embolization for the management of unruptured cerebral aneurysms: Report of six cases. *Acta Neurochir (Wien)*. 2009;**151(12)**:1663–1672.

7. Klisch J, Clajus C, Sychra V, Eger C, Strasilla C, Rosahl S, et al. Coil embolization of anterior circulation aneurysms supported by the Solitaire AB Neurovascular Remodeling Device. *Neuroradiology*. 2009;**52(5)**:349–359.

8. Klisch J, Eger C, Sychra V, Strasilla C, Basche S, Weber J. Stent-assisted coil embolization of posterior circulation aneurysms using Solitaire AB: Preliminary experience. *Neurosurgery*. 2009;**65(2)**:258–266.

9. Rohde S, Bendszus M, Hartmann M, Hahnel S. Treatment of a wide-necked aneurysm of the anterior cerebral artery using two Enterprise stents in "Y"-configuration stenting technique and coil embolization: A technical note. *Neuroradiology*. 2009;**52(3)**:231–235.

10. Pumar JM, Lete I, Pardo MI, Vazquez-Herrero F, Blanco M. LEO stent monotherapy for the endovascular reconstruction of fusiform aneurysms of the middle cerebral artery. *AJNR Am J Neuroradio*l. 2008;**29(9)**:1775–1776.

11. Dorn F, Niedermeyer F, Balasso A, Liepsch D, Liebig T. The effect of stents on intra-aneurysmal hemodynamics: In vitro evaluation of a pulsatile sidewall aneurysm using laser Doppler anemometry. *Neuroradiology*. 2010;**53(4)**:267–272.

12. Lylyk P, Miranda C, Ceratto R, Ferrario A, Scrivano E, Luna HR, et al. Curative endovascular reconstruction of cerebral aneurysms with the pipeline embolization device: The Buenos Aires experience. *Neurosurgery*. 2009;**64(4)**:632–642.

13. Yamada NK, Cross DT 3rd, Pilgram TK, Moran CJ, Derdeyn CP, Dacey RG Jr. Effect of antiplatelet therapy on thromboembolic complications of elective coil embolization of cerebral aneurysms. *AJNR Am J Neuroradiol*. 2007;**28(9)**:1778–1782.

Case Report

The Vanishing Veins: Difficult Venous Access in a Patient Requiring Translumbar, Transhepatic, and Transcollateral Central Catheter Insertion

Yazmin Yaacob[1], Rozman Zakaria[1], Zahiah Mohammad[1], Ahmad Razali MD Ralib[2], Ahmad Sobri Muda[1]

Submitted: 4 Feb 2011
Accepted: 29 May 2011

[1] *Department of Radiology, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia*

[2] *Department of Radiology, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200 Kuantan, Pahang, Malaysia*

Abstract

Central venous catheter placement is indicated in patients requiring long-term therapy. With repeated venous catheterisations, conventional venous access sites can be exhausted. This case illustrates the expanding role of radiology in managing difficult venous access. We present a case of translumbar, transhepatic, and transcollateral placement of central catheter in a woman with a difficult venous access problem who required lifelong parenteral nutrition secondary to short bowel syndrome. This case highlights the technical aspects of interventional radiology in vascular access management.

Keywords: *central nervous system neoplasms, child, oncology, recurrence, rhabdoid tumour, teratoma*

Introduction

Central venous catheter placement is indicated in patients requiring long-term parental nutrition or intravenous drug therapy. Long-term dependency on central catheters predisposes patients to complications such as central vein stenosis or thrombosis. Inevitably, access to common venous sites such as the femoral and neck veins becomes impossible. An interventional radiology unit that is properly equipped with imaging guidance knowledge and catheter skills can be a saviour in treating these patients.

Case Report

Here, we report a case of a 39-year-old woman with history of colon carcinoma complicated by multiple laparotomies as well as adhesion colic for the past 8 years. Secondary to multiple small bowel resections, she developed short bowel syndrome, requiring long-term total parental nutrition. She had implantable venous ports and percutaneous central cannulations inserted through the jugular, femoral, and subclavian veins. She then developed central vein stenosis and collateral formation of the upper body, resulting in minimal access to conventional venous sites. We decided to insert a subcutaneous venous port into the inferior vena cava (IVC) using the translumbar approach for vascular access. However, the port only lasted for 5 months due to line-related sepsis. Computed tomography (CT) examination also revealed a stenotic infrarenal IVC post–translumbar catheter insertion. Our next option was to insert a central venous catheter via the transhepatic route. The transhepatic venous access remained functional over the next 4 months. Lastly, we successfully inserted a central cannula from the left basilic vein via a large collateral that reached the superior vena cava. However, the central cannula only lasted for another 3 months. In total, we managed to provide venous access using the percutaneous approach with image guidance and provided vascular access for at least a year before surgical intervention. These were the techniques used to provide central venous access for the patient.

Translumbar access

The procedure was performed under CT guidance using aseptic technique. CT fluoroscopy guidance was used to puncture the IVC. The patient was positioned prone with a 15- to 20-degree

elevation on the right side. A temporary entry site was marked. A short scanning range, targeting the infrarenal IVC segment, was performed to detect a suitable entry site. The vital signs were monitored. After the location of the IVC puncture site was determined, local anaesthetic was infiltrated into the skin and the subcutaneous tissue. A small incision was made with a scalpel.

We used the AccuStick II introducer system (Boston Scientific, Natick, MA). A 21-gauge, 15-cm diagnostic needle with a stylet was used to puncture the IVC. The anterolateral margin of the vertebra was used as the directional landmark. The needle was advanced cephalad and medially at the level of L2 and L3 below the renal veins (Figure 1). A successful puncture of the IVC was confirmed by CT fluoroscopy and free aspiration of blood from the IVC. A nitinol 0.018-inch guide wire was manoeuvred into the needle under intermittent fluoroscopy guidance and advanced well into the IVC. The introducer needle was exchanged with a 6 Fr co-axial vascular sheath that was introduced over the 0.018-inch wire. A 0.035-inch guide wire, 150 cm in length, was advanced into cavo-atrial junction.

The port was implanted just above the anterior superior iliac spine, which was approximately 10 to 15 cm lateral to the entry site. Local anaesthetic was injected from the port site along the tunnelling pathway towards the entry site. The catheter was a 10 Fr single lumen (B Braun® subcutaneous port set) silicone rubber catheter, which was tunnelled subcutaneously and brought to the entry site.

With the guide wire placed within the IVC, the tract was progressively dilated. A 10 Fr, 16 cm peel-away vascular sheath, which was part of the set, was inserted. For an ideal translumbar catheter insertion, a separate peel-away sheath with a recommended length between 18 and 20 cm is needed given the distance between the tissue and the entry site. However, this was not available at the time of the procedure. The peel-away sheath facilitated the insertion of the catheter into the IVC after catheter measurement was performed. The peel-away sheath was removed, and the final position of the catheter tip was confirmed with CT fluoroscopy. The proximal end of the catheter was then attached to the port and anchored to the adjacent muscle layer. The incision was closed with double-layer absorbable sutures. Similarly, the entry site was also sutured (Figure 2).

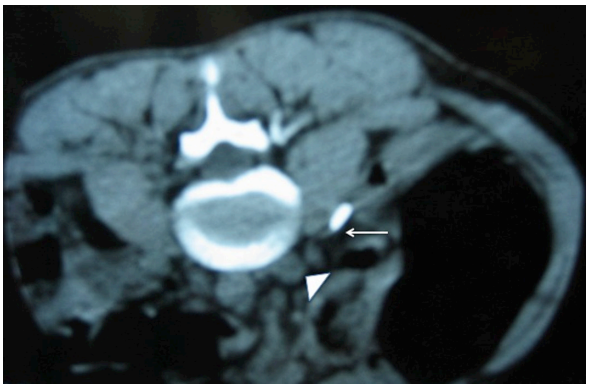


Figure 1: Computed tomography fluoroscopy showing the puncture needle (white arrow) approaching the infrarenal inferior vena cava (white arrowhead).

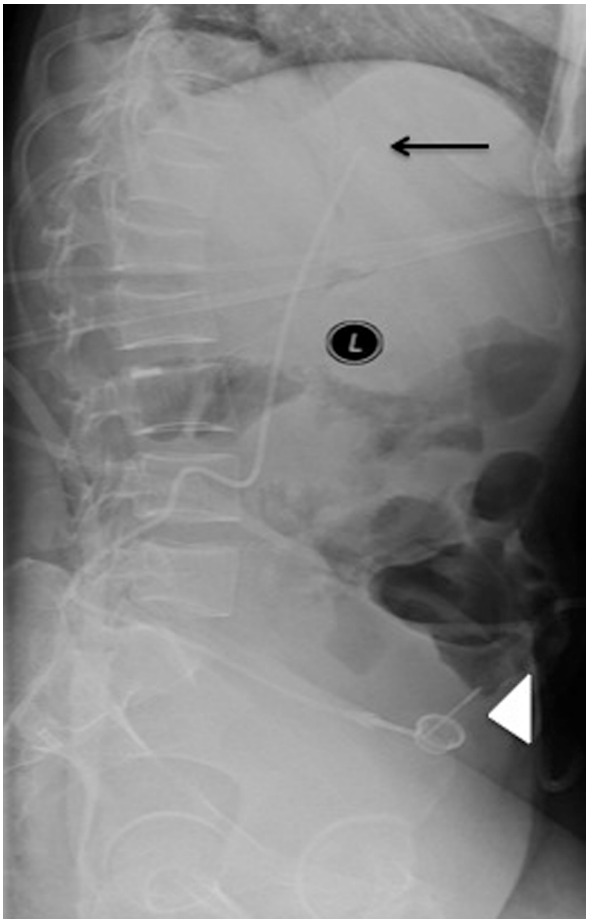


Figure 2: Lateral radiograph showing the translumbar chemoport. The tip of the catheter is near the cava-atrial junction (black arrow), and the subcutaneous port is seen anterolaterally (white arrowhead).

Transhepatic access

The transhepatic access was easier in comparison to the translumbar approach. The transhepatic route can be achieved with a 22G Chiba needle, as in percutaneous cholangiography under fluoroscopy. However, the aim is to reach the hepatic veins instead of the biliary tree. Another alternative, which we used in this patient, is a direct puncture with ultrasound guidance. After the hepatic vein was identified, a 0.018-inch wire was advanced to the IVC. In our patient, we used the AccuStick II (Boston Scientific) system to exchange the microwire to a 0.035-inch hydrophilic guide wire. The tip of the guide wire was placed at the distal superior vena cava. Similarly, subsequent dilatation and insertion of an appropriately sized peel-away sheath was advanced to the cavo-atrial junction. The external portion of the catheter was then tunnelled subcutaneously in the anteroinferior direction (Figure 3).

Transcollateral access

The patient had documented angiographic evidence of central vein stenosis from previous catheterisations of the jugular and subclavian veins (Figure 4). Our approach was via the left basilic vein; a 4 Fr vascular sheath was inserted, and a 4 Fr Cobra catheter was used to aid manoeuvring. Because multiple collaterals were observed, we chose the largest venous collateral and the most direct route to the superior vena cava. We achieved the catheter placement with multiple attempts and sheer persistence. The catheter was manoeuvred over the wire with the tip placed within the distal superior vena cava (Figure 5).

Discussion

Central venous access in a patient with central vein stenosis remains a challenge for both the referring physicians and the interventional radiologists. Non-conventional methods have been developed, as described above. Among all of the aforementioned techniques, translumbar catheter insertion using CT-guided fluoroscopy appears to be the best option, in our opinion. Kenney et al. (1) first described the technique for translumbar placement for central venous access in 1985. We used CT fluoroscopy for entry into the IVC. Conventionally, the IVC can be accessed with normal C-arm fluoroscopy either with or without a marker from a pigtail catheter or a guide wire placed in the IVC from the femoral venous approach. We found that it was easier to locate

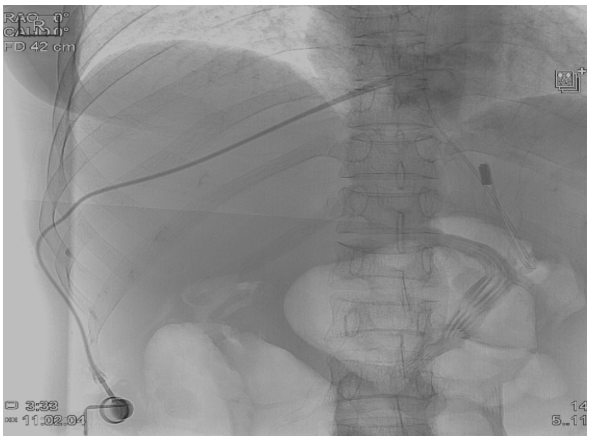


Figure 3: Frontal radiograph showing the transhepatic subcutaneous port placement of the patient.

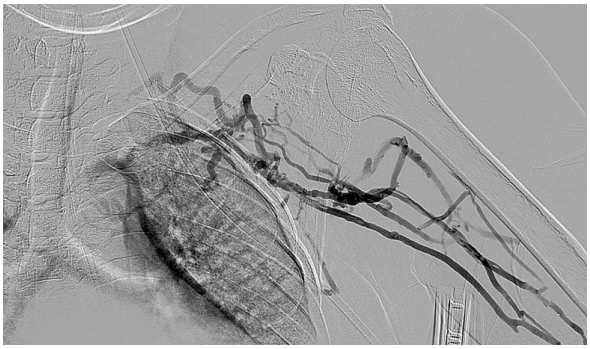


Figure 4: Left upper limb venogram showing central vein stenosis with collateral formation.

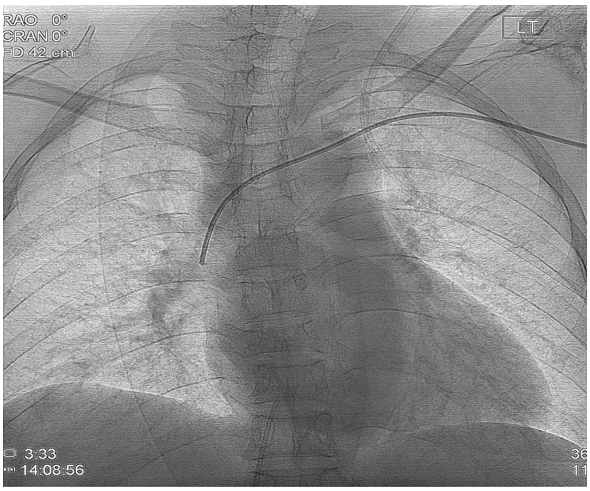


Figure 5: Fluoroscopy image showing the peripherally inserted central catheter, which was treaded across a collateral vein.

the IVC using CT fluoroscopy. The patency of the IVC also can be assessed concurrently. During puncturing, the direction of the needle can be monitored without over-shooting to the adjacent structures, particularly the ureters and the aorta. Once the IVC was punctured, it was easy to place the distal tip of the guide wire at the junction of the IVC and the right atrium. However, CT-guided puncture is associated with higher radiation doses and, in certain circumstances, necessitates the use of an angiographic fluoroscopy machine that requires patient transfers. Newer versions of CT fluoroscopy have lower radiation doses with more protection devices for the operators. In this patient, we used a single-lumen chemoport for hyperalimentary nutrition. The translumbar venous access can also be successfully used and is suitable for a dual-lumen haemodialysis catheter (2,3). Additionally, it should be noted that although radiation is a concern, CT-guided insertion is safer than conventional fluoroscopy guidance (4). Resuscitation is difficult in these patients because they have limited venous access to begin with, should there be any fatal event.

Transhepatic catheterisation, which was first described by Po et al. (5) in 1994, is a well-known technique for central venous access. It was technically easier compared with the translumbar approach but presented a number of possible complications, such as bleeding, catheter dysfunction, and biliary-related complications. These complications had been known to be mainly associated with larger catheters intended for haemodialysis (6,7).

Transcollateral access is technically challenging because the route of a collateral is usually tortuous. Retrospectively, we feel that this approach should have been tried first to preserve the IVC. It appears to be safe and effective, and also provided our patient with a reliable venous access for three months (8–10).

Non-conventional image-guided percutaneous catheter placements are the exception and are requested only when traditional access sites are unavailable. They are good alternative routes for permanent venous access, and with proper technique and care, will be associated with minimal post procedural complications. Proper anatomical, radiological, and technical knowledge will influence the success of the procedures, which are best performed by a trained interventional radiology unit, as illustrated in the case.

Authors' Contributions

Conception and design: ASM
Collection and assembly of the data, final approval of the article: YY
Drafting of the article: YY, RZ, ZM
Critical revision or the article: ARMR, YY

Correspondence

Dr Yazmin Yaacob
MBChB (Otago), MMed Radiology (UKM)
Department of Radiology
Universiti Kebangsaan Malaysia Medical Centre
Jalan Yaacob Latif, Bandar Tun Razak
56000 Cheras
Kuala Lumpur, Malaysia
Tel: +6012-201 0582
Email: minyaacob@yahoo.com

References

1. Rajan DK, Croteau DL, Sturza SG, Harvill ML, Mehall CJ. Translumbar placement of inferior vena caval catheters: A solution for challenging hemodialysis. *Radiographics*. 1998;**18(5)**:1155–1167.

2. Denny DF Jr, Greenwood LH, Morse SS, Lee GK, Baquero J. Inferior vena cava: Translumbar catheterization for central venous access. *Radiology*. 1989;**172(3 Pt 2)**:1013–1014.

3. Lund GB, Lieberman RP, Haire WD, Martin VA, Kessinger A, Armitage JO. Translumbar inferior vena cava catheters for long-term venous access. *Radiology*. 1990;**174(1):**31–35.

4. Kariya S, Tanigawa N, Kojima H, Komemushi A, Shomura Y, Ha-Kawa SK, et al. Percutaneous translumbar inferior vena cava cannulation under computed tomography guidance. *Jpn J Radiol*. 2009;**27(4)**:176–179.

5. Po CL, Koolpe HA, Allen S, Alvez LD, Raja RM. Transhepatic PermCath for hemodialysis. *Am J Kidney Dis*. 1994;**24(4)**:590–591.

6. Smith TP, Ryan JM, Reddan DN. Transhepatic catheter access for hemodialysis. *Radiology*. 2004;**232(1)**:246–251.

7. Yap DY, Tso WK, Chu FS, Chan TM, Lai KN, Tang SC. Transhepatic catheter placement of haemodialysis catheter: A solution for vascular access exhaustion. *Nephrology (Carlton)*. 2010;**15(6)**:661–662

8. Forauer AR, Brenner B, Haddad LF, Bocchini TP. Placement of hemodialysis catheters through dilated external jugular and collateral veins in patients with internal jugular vein occlusions. *Am J Roentgenol*. 2000;**174(2)**:361–362.

9. Teichgraber UK, Streitparth F, Gebauer B, Benter T. Placement of a port catheter through collateral veins in a patient with central venous occlusion. *Cardiovasc Intervent Radiol.* 2010;**33(2)**:417–420.

10. Powell S, Chan TY, Bhat R, Lam K, Narlawar RS, Cullen N, et al. A retrospective comparative study of tunneled haemodialysis catheters inserted through occluded or collateral veins versus conventional methods. *Cardiovasc Intervent Radiol.* 2010;**33(4)**:744–750.

Guideline For Authors

The MJMS welcomes manuscripts on all aspects of medicine/health science from any part of the world. We are members of World Association of Medical Editors (WAME) and Council of Science Editors (CSE).

Manuscripts must be submitted in English. Manuscripts are considered for publication in MJMS with the understanding that they have not been published or submitted for publication elsewhere. The manuscript should be submitted to the Editor, Professor Jafri Malin Abdullah via Manuscript Central http://mc.manuscriptcentral.com/maljms. Please note that at the moment, we do not accept Miscrosoft Word 2007 documents (*.docx). Please use Ms Word's "Save As" option to save your document as an older (.doc) file type
The guidelines listed below are in accordance with the Uniform Requirement for Manuscripts Submitted to Biomedical Journals (October 2008 revision) of the International Committee of Medical Journal Editors.

Forms

When submitting manuscripts, authors are required to sign the Authorship Agreement Form, Patient Consent Form (if the manuscript includes identifiable patients) and the Publication Agreement Form.

Types of Manuscripts

MJMS publishes the following types of manuscripts.

Editorials (E): Brief, substantiated commentary on subjects of topical interest.
Abstracts: Not required
Text: Not more than 1200 words (excluding references and figure/table legends)
Tables and figures: Not more than 1
References: Not more than 20

Original Article (OA): Reports of original clinical or investigative laboratory research.
Abstract: Not more than 275 words
Text: Not more than 3500 words (excluding references and figure/table legends)

Review Article (RA): A review article aims to give an overview of a particular subject suitable for a wide audience. Review articles should be recent rather than a historical review of the article on the topic.
Abstract: Unstructured Abstract not more than 275 words
Text: Not more than 4500 words (excluding references and figure/table legends)
References: Not more than 80

Case Report (CR): Brief case reports of unusual interest.
Abstract: Unstructured abstract, not more than 175 words
Text: Not more than 2000 words
References: Not more than 10
Figures and tables: Less than 3 figures and tables

Brief Communications (BC): A Brief Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus.
Abstract: Not more than 175 words
Text: Not more than 1500 words
References: Less than 20
Figures and tables: Not more than 3

Special communications (SC): These manuscripts describe an important issue in clinical medicine, public health, health policy, or medical research in a scholarly, thorough, well-referenced, systematic or evidence-based manner.
Abstract: A narrative (unstructured) abstract of 200 words or fewer is required
Text: Not more than 3000 words (excluding tables, figures, or reference
References: Less than 80

Letter to the Editor (LE): Comments on articles published within 6 months in MJMS or articles of interest to the biomedical community
Text: Should not exceed 500 words
References: Not more than 6 references

Letters in reply (LR): Replies by authors
Text: should not exceed 500 words
References: Not more than 6

Extended abstracts (EA): Only published as supplementary issues during conferences. Extended abstracts are also peer reviewed. Authors who intend to submit extended abstracts should contact directly via email at mjms.usm@gmail.com.

Ethical requirements

In experiments on human subjects, authors should mention whether the methods followed were in agreement with the ethical standards of the responsible committee (institutional and national) and the Declaration of Helsinki (October 2008 revision). Similarly, the use of animals in research must conform to the institutional and national guidelines.

Patient consent

The author must provide the Malaysian Journal of Medical Sciences with a written consent signed by the patient, or the patient's parents/legal guardian, when submitting a patient video or photograph in which a patient is identifiable (See Patient Consent Form). This form can be downloaded from our website.

Style manuscript and Format

Text: Use subheadings for long articles and double-space all portions of the manuscript

Title page: The title page should have the following information:

i. Article title without abbreviation
ii. Authors' names and institutional affiliations: Full names are required, indicate last name with SMALL CAPS. For example, Mohammed Ali JAMALUDDIN.
iii. Contact information for corresponding authors. The name, address, email of one author who is responsible for all communication concerning the manuscript are required.

Abstract: The length of abstract depends on the type of manuscript submitted. The abstract should state the purpose of the study, a brief description of the procedures employed, main findings and principal conclusions. Abbreviations, foot notes, references and subheadings should be avoided. For original articles, the abstract format is structured as background, methods, results, and conclusion. For other articles, the abstract format is unstructured.

Keywords: Authors must provide between three and six keywords that characterize the main topics of the article. Use recognized vocabularies related to the discipline discussed, such as the MeSH thesaurus http://www.nlm.nih.gov/mesh/MBrowser.html. We encourage the use of synonyms for terms provided in the article title, this is to aid database searches.

Tables: Tables must be numbered sequentially and in the order in which they are mentioned in the text. Tables must have brief descriptive title. Preferably, tables must be prepared according to the guides in Chicago Manual of Style.

Figures: Figures must be numbered sequentially and in the order in which they are mentioned in the text. Figure legends are needed for all figures. Grayscale and color artwork should have a minimum resolution of 300 dpi.

Videos: We also welcome submission of short videos as supplementary file. Videos may be useful for demonstrating complex laboratory, surgical or medical procedures. The demonstration of the experiment must be shown in orderly fashion, including a demonstration of quipment and reagent. Researchers should be properly attired when handling animals, reagents and chemicals. Each video file must be under 5 minutes. We accept .mov, .avi, .swf formats. The video should make a specific point; particularly, it should demonstrate the features described in the text of the manuscript. Special effects or text are not permitted to be inserted in the video. Unfortunately, we do not do video editing and production.

Reference: References should be numbered consecutively in the order in which they are first mentioned in the text (citation-sequence)—the Vancouver style. Identify references in text, tables and legends by Arabic numerals in parentheses. For formatting end references, we recommend following the guidelines of the Council of Science Editors (CSE) which can be accessed through http://library.duke.edu/research/citing/workscited/

Journal article: The titles of journal should be abbreviated according to the style used in http://www.ncbi.nlm.nih.gov/sites/entrez?Db=journals&Cmd=DetailsSearch&Term=currently indexed[All] or http://www.efm.leeds.ac.uk/~mark/ISIabbr/A_abrvjt.html

> Theriault A, Caao JT, Gapor A. Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecule and adhesion to monocytes. *Atherosclerosis*. 2002; **160(1)**:21-30.

If there are more than six authors, list the first six authors and use "et al." for the subsequent authors.

Books: Authors' surname Initials. *Title of book*. # ed. [if not 1st]. Place of publication. Publisher's name; Year of publication.

> Carlson BM. *Human embryology and developmental biology*. 3rd ed. St Louis: Mosby; 2004.

Online article: Journal articles in electronic format: Authors' surname Initials. Title of article. Abbreviated title of journal [Internet]. Year of publication [cited YYYY MMM DD]. Available from: URL.

> Rabbani SI, Devi K, Khanam S. Role of pioglitazone with metformin or glimepiride on oxidative stress-

induced nuclear damage and reproductive toxicity in diabetic rats. *Malaysian J Med Sci* [Internet]. 2010 [cited 2010 Mar 21];**17(1)**:3–11. Available from: http://ernd.usm.my/journal/journal/02-171OA1pioglitazone.pdf

For other forms of reference, please refer to the National Library of Medicine http://www.nlm.nih.gov/pubs/formats/recommendedformats.html

Editing

A manuscript may be corrected for length, grammatical correctness, sentence structure and journal style. Accepted manuscripts are edited in accordance with the *CSE Manual of Style, 7th edition* and *Chicago Manual of Style 15th edition*. The final proof of the manuscript will be sent to the corresponding author for final checking. The author should not make any changes to the contents of the manuscript at this stage.

Editorial policies for authors

Authors are required to sign the Authorship Agreement Form when submitting a manuscript to MJMS. In addition, authors are required to identify their contributions to the work described in the manuscript. If requested to see the original data, authors must provide the data and must cooperate in obtaining and providing the data on which the manuscript is based.

Conflicts of Interest and Financial Disclosures

A conflict of interest may arise when an author (or the author's institution or employer) has financial or personal relationships that could influence the author's decisions, work, or manuscript. All authors are required to disclose all potential conflicts of interest, including specific financial interests and relationships and affiliations (other than those affiliations listed in the title page of the manuscript) relevant to the subject of their manuscript. Please refer to the Authorship Agreement Form.

Authors are expected to provide detailed information about all relevant financial interests and relationships or financial conflicts within the past 5 years and for the foreseeable future, particularly those present at the time the research was conducted and through publication, as well as other financial interests (such as patent applications in preparation), that represent potential future financial gain. Authors may do so in the covering letter submitted via Manuscript Central.

Funding/Support and Role of Sponsor

All financial and material support for the research and the work should be clearly and completely identified in an Acknowledgment section of the manuscript. The specific role of the funding organization or sponsor in each of the following should be specified: "design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

References

1. Council of Science Editors, Scientific Style and Format. The CSE Manual for Authors, Editors and Publishers. 7th ed. Reston (VA): The Council; 2006.
   Council; 2006.
2. The Chicago manual of style. 15th ed. Chicago: The University of Chicago Press; 2003.
3. Uniform Requirements for Manuscripts Submitted to biomedical journals: Writing and Editing for biomedical publication [Internet]. International Committee of Medical Journal Editors: 2009. Available from: http://www.icmje.org/

Authorship Agreement Form

Date: .....

Manuscript title: .....

The completed forms must be mailed to the Editor, Malaysian Journal of Medical Sciences, USM Press, c/o School of Medical Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, 16150 Kota Bharu, Malaysia, or faxed to +609 767 2359

Ethical Requirement

The authors attest that full and informed consent was obtained from human subjects and that the work was performed in accordance with the humane and ethical principles of research outlined in the Helsinki guidelines. In countries where institutional review is established, this statement also attests to the approval of that institution of the protocol for all aspects of the investigation presented in a manuscript involving humans or animals.

Conflict Of Interest And Financial Support

The undersigned authors agree that any financial interests that exist for individual contributors in connection with this manuscript have been disclosed in the covering letter submitted through Manuscript Central. Sources of financial support of the project are named in the covering letter as well as in the Acknowledgements.

Duplicate Publications

The undersigned Author(s) certify that neither this manuscript nor one with substantially similar content under their authorship has been published or being considered for publication elsewhere in any language. They also certify that any previous presentations of this paper in meetings are mentioned in the covering letter.

Authorship

Author(s) attest that all persons designated as authors qualify for authorship and all those who qualify are listed. All others who contributed to the work but are not authors (if any) are named in the Acknowledgements of the manuscript.

In the space marked "Contribution Codes", authors should mark those code letters from the box that designate their own substantive contribution(s) to the paper.

Contribution codes

A: Conception and design
B: Analysis and interpretation of the data
C: Drafting of the article
D: Critical revision of the article for important intellectual content
E: Final approval of the article
F: Provision of study materials or patients
G: Statistical expertise
H: Obtaining of funding
I: Administrative, technical, or logistic support
J: Collection and assembly of data

Names of all authors

Contribution codes*	Author's Name	Signature

Patient Consent Form

I give my permission for the following material to appear in the print and online versions of the Malaysian Journal of Medical Sciences. The completed forms must be mailed to the Editor, Malaysian Journal of Medical Sciences, USM Press, c/o School of Medical Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, 16150 Kota Bharu, Malaysia, faxed to +609 767 2359, or emailed to mjms.usm@gmail.com

Title or subject of article, photograph or video:

I understand that my name will not be published but that complete anonymity cannot be guaranteed.

Please check the appropriate box below after reading each statement.

☐ I have read the manuscript or a general description of what the manuscript contains and reviewed all photographs, illustrations, or video files (if included) in which I am included that will be published.

☐ I have been offered the opportunity to read the manuscript and to see all photographs, illustrations, or video files (if included) in which I am included, but I waive my right to do so.

Signature of patient or guardian : .....
Name : .....
Date : .....

Copyright Transfer Form

The Universiti Sains Malaysia Press ("USM Press") is pleased to undertake the publication of your work tentatively titled _____ _____(the "Contribution") in its publication _____ (the "Publication").

You, the undersigned individual(s), will retain copyright to the Contribution as author(s); and you grant us, as publisher, the limited rights detailed below, which are expressly conditioned on the terms of this agreement.

We both acknowledge that a static agreement may not be able to contemplate all possible licensing arrangements, technologies or future developments, and therefore we both agree to cooperate in good faith to achieve our mutual goals of maximizing dissemination of your Contribution while ensuring the sustainability of scholarly publishing.

Accordingly, we submit the following terms of publication for your consideration.

1. ***License grant***
   You grant to USM Press a worldwide, royalty-free, non-exclusive license to:
   (i) reproduce, publicly display, publicly perform and distribute the Contribution in the Publication;
   (ii) authorise third party users of the Publication in electronic or digital (or other intangible) form to download and print out copies of the Contribution for their personal or internal institutional use;
   (iii) authorize others to reproduce, publicly display, publicly perform and distribute the Contribution as part of the Publication through any distribution channels and in any media or format through which USM Press may distribute or make available substantially all the contents of the Publication; and
   (iv) deposit with or otherwise make the Contribution available to digital repositories as required by your funding sources upon your request.

   The above rights may be exercised in all media and formats, whether now known or hereafter devised, [and in all languages] in which the Publication as a whole (or substantially all its contents) may be distributed, whether or not the Contribution may be individually or partially accessed or retrieved. The above rights include the right to edit [and translate] the Contribution and to make such modifications as are technically necessary or desirable to exercise these rights in differing media and formats.

2. ***Exclusive rights***
   You grant to USM Press the worldwide, royalty-free, exclusive right to first publish the Contribution.

3. ***Author Rights***
   You retain the rights to
   (i) reproduce and distribute a reasonable number of copies of any version of the Contribution, including but not limited to the published version, or portions or derivative works thereof, in the course of your teaching, research, conference presentations and similar professional, scientific, or academic activities (but not permit commercial publication or widespread distribution of the Contribution or any significant portion or derivative work thereof);
   (ii) post or otherwise make any version of the Contribution, or portions or derivative works thereof, available on your personal web site; and
   (iii) [if and as required by your employing institution(s) or your funding source(s),] make any version of the Contribution available on digital repositories; provided in each case [acronym/abbreviation] (and Publication) is cited as the first/forthcoming publisher of the Contribution and you accurately distinguish any modified version of the Contribution from that published or to be published by us.

4. ***Editing***
   USM Press will make no material modification to the content of the Contribution without your consent. The Contribution will also be subjected to editing for language clarity and conciseness, should we deemed it necessary. However, if you fail to return the edited manuscript or proofs of the Contribution by the reasonable deadline set by us, you will be deemed to have consented to that modified version.

5. ***Credit***
   USM Press agrees to make commercially reasonable efforts to include (and require its sublicensees to include) appropriate credit to the author(s) in customary placement with every copy or use of the Contribution as described in Sections 1 and 2.

6. ***Warranties***
   You warrant that
   (i) you are the creator of the Contribution or otherwise have the rights necessary to grant the licenses granted to USM Press herein; and
   (ii) the Contribution contains no material that infringes or violates any intellectual property or contractual rights of others or that constitutes defamation or invasion of privacy. The foregoing warranties apply only to the Contribution in the form submitted by you to USM Press, and not to any modifications or additions made by USM Press, reviewers or other third parties.

7. ***Termination***
   If we do not publish the Contribution in the Publication within [twelve (12)] months following submission of the Contribution, extended to the extent of any delays caused by you, you may terminate this agreement and the licenses granted to us hereunder by providing us with written notice of termination, unless we publish the Contribution within [three (3)] months following receipt of such notice. Such termination of this agreement will be your exclusive remedy for any failure by us to publish the Contribution.

8. ***Miscellaneous***
   This agreement contains the entire understanding of the parties and will be governed by the laws of the State of [state], without reference to its conflicts of laws principles. This agreement can only be modified by an agreement in writing signed by the parties. This agreement may be executed in one or more counterparts (including, without limitation, by facsimile or .pdf signature) each of which will be deemed an original and all of which will be taken together and deemed to be one instrument. USM Press may assign this agreement in connection with a sale of the Publication or a sale, merger or reorganization of our organization.

If the foregoing terms are acceptable, please sign and date this agreement. All joint authors must sign. Please return the original to USM Press immediately and retain a copy for your records.

Corresponding author name (print or type):	
Address:	**Author signature:** **Date:**

Joint author name (print or type):	
Address:	**Author signature:** **Date:**

Joint author name (print or type):	
Address:	**Author signature:** **Date:**

Joint author name (print or type):	
Address:	**Author signature:** **Date:**

Joint author name (print or type):	
Address:	**Author signature:** **Date:**

Joint author name (print or type):	
Address:	**Author signature:** **Date:**

The completed form should be scanned/emailed to mjms.usm@gmail.com or alternatively, faxed to +609-767 2359.

Subscription Form

Name : .....
Address : .....
.....
.....
Tel : .....
Fax : .....
Email : .....
Date : .....

I would like to subscribe copy/copies of the following:

Volume 18, No. 4, 2011	MYR30 (within Malaysia)	Quantity: .....
	USD30 (International)	

* Previous issues are available on request and are subjected to availability.

Payment should be made in Malaysian Ringgit or US Dollar.
Methods of payment are as follows:

Crossed Cheque:

Made payable to
BENDAHARI UNIVERSITI SAINS MALAYSIA
Address
The Malaysian Journal of Medical Sciences
Penerbit Universiti Sains Malaysia
Universiti Sains Malaysia
11800 USM Pulau Pinang
MALAYSIA

Bank:

Payment may be made through banks to
Bank name: Bank Muamalat Malaysia Berhad
Branch: USM
Account number: 0702-0001054-71-6
Swift Code: BMMBMYK1

For more information, please contact Journal Division, Penerbit Universiti Sains Malaysia, by telephone at +604-6534423, by fax at +604-6575714, or by email at journal@usm.my.