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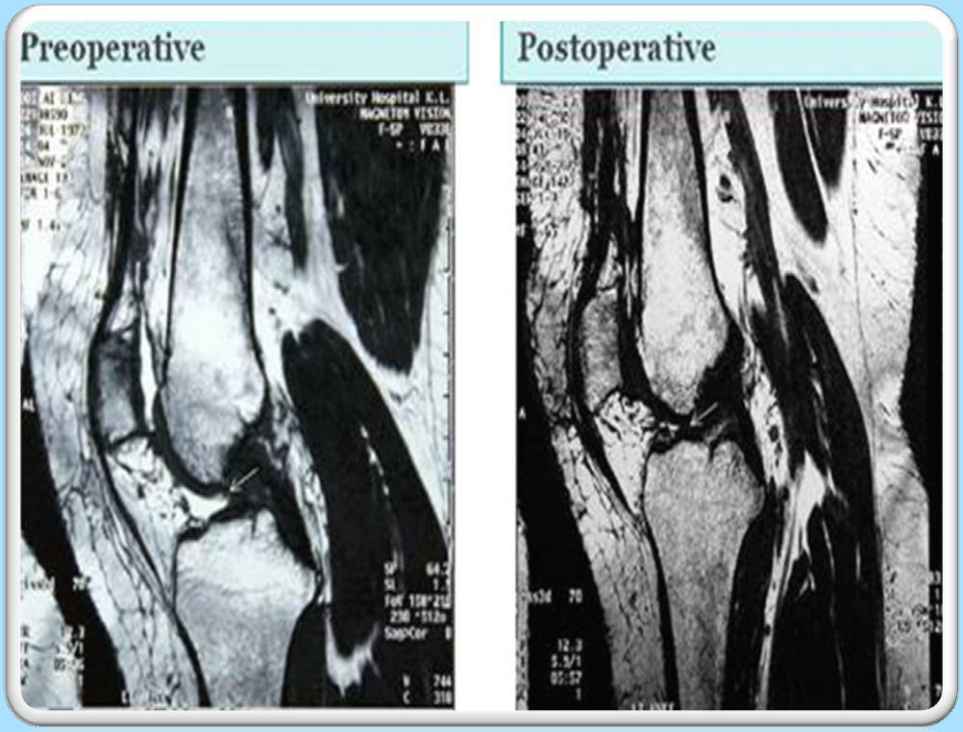

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JUMMEC publishes both basic and applied science as well as clinical research studies on any area of medicine that is of interest and relevance to the medical community. This is a peer-reviewed Journal that publishes twice yearly on Review

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We welcome journal submissions throughout the year but preferably by **March** and **September**. Articles submitted for publication are understood to be offered only to *JUMMEC* and which have not been sent to other journals for consideration.

Cover

Magnetic resonance images obtained 3 year after autologous chondrocyte implantation preoperatively and postoperatively. Image courtesy of P. N. Shilpa.

Instructions for Authors

The Journal of Health and Translational Medicine (JUMMEC) publishes both basic and applied science as well as clinical research studies on any area of medicine that is of interest and relevance to the medical community. This is a peer-reviewed journal that publishes Reviews Articles, Original Articles, Short Communications, Clinico-pathological Conference Abstracts, Case Reports, Letters to the Editor and Book Reviews.

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Foreword from the Editor

Dear Readers,

It is my great pleasure to present the first issue of JUMMEC for 2014. This issue comprises four articles, including two review papers, covering a varied scope of interesting and informative topics in health and translational medicine.

On behalf of the Editorial Board, I would like to thank the authors who have submitted articles to JUMMEC. I hope this would encourage many more authors to submit their manuscripts and publicise their research findings or reviews of topics of interest. In addition, I would like to thank the sub-editors and reviewers who have contributed in one way or another to the publishing of this issue, and whose work has helped enhanced the quality of the articles. Despite that there were cases in which manuscripts were rejected; our motivation has always been to make publications possible.

The first article of this issue, authored by Jindal *et al.*, reviews the challenges in antimicrobial peptide studies. The authors identify crucial physicochemical parameters of the peptides that determine their activities. This is important for studies designing new synthetic antimicrobial peptide analogues.

The second article was written by a group of orthopaedic surgeons and scientists reporting on the autologous chondrocyte implantation procedure in the treatment of knee focal cartilage defects. The treatment led to a favourable outcome in focal cartilage defects involving femoral condyle. The study, performed by Abbas *et al*., showed improvement in tissue repair which was maintained until three years follow-up in these cases.

In the third article, results of a survey investigating factors that contribute to the low quantity of organ donation in Malaysia are reported. In their article, Tumin *et al.* identifies public awareness as the main contributing factor that should be addressed by the authorities to mitigate the problem of organ shortage in Malaysia.

This issue of JUMMEC is rounded off by a comprehensive review article on anti-retroviral therapy, which was made available since 1996. In this article, Lubis and Bulgiba provide updates and discuss diverse issues pertaining to the lifelong therapy that has benefited millions of HIV-infected patients throughout the world.

May this brief overview of the various articles of this issue arouse our readers' interest and inspire submission of more articles that may be published in future issues of JUMMEC.

With best wishes,

Onn Hashim
Editor
JUMMEC – the Journal of Health and Translational Medicine

NET CHARGE, HYDROPHOBICITY AND SPECIFIC AMINO ACIDS CONTRIBUTE TO THE ACTIVITY OF ANTIMICROBIAL PEPTIDES

Jindal MH, Le CF, Mohd Yusof MY, Sekaran SD

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur.

Correspondence:
Prof. Dr. Shamala Devi Sekaran
Department of Medical Microbiology,
Faculty of Medicine,
University of Malaya, 50603 Kuala Lumpur.
Email: shamalamy@yahoo.com

ABSTRACT

Antimicrobial peptides (AMPs) have gained increasing attention as a potential candidate in the development of novel antimicrobial agent. Designing AMPs with enhanced antimicrobial activity while reducing the cell toxicity level is desired especially against the antibiotic-resistant microbes. Various approaches towards the design of AMPs have been described and physicochemical properties of AMPs represent the primary factors determining the antimicrobial potency of AMPs. The most common parameters include net charge and hydrophobicity, which greatly influence the antimicrobial activity of AMPs. Moreover, certain amino acids would have critical importance in affecting the antimicrobial activity as well as cell cytotoxicity of AMPS. In this review, net charge, hydrophobicity, and specific amino acid residues were discussed as factors contributing to the antimicrobial activity of AMPs.

Keywords: *Antimicrobial peptides, antibiotic-resistant, net charge, hydrophobicity*

Introduction

Living organisms are constantly interacting with other life forms in their surrounding complex environments, which include potential harmful challenges by other pathogenic life forms. The host's first line of immune defense must be able to recognize and destroy any potential invasion efficiently. It is desirable to have the pathogens eliminated without the unnecessary activation of the secondary immune mechanism to prevent overwhelming immune responses in the host (1).

Antimicrobial peptides (AMPs) are an essential innate immune component produced by a wide range of multicellular organisms (2). AMPs are relatively small in size (generally 12–50 amino acid residues long), possess multiple cationic amino acids with an overall net charge ranging from +2 to +9, and are amphipathic (containing ~50% hydrophobic residues) (3). AMPs can be classified into four major classes, namely, β-sheet structures stabilized by two or three disulphide bridges, α-helices, extended helices (polyprohelices) with a predominance of one or more amino acids, and loop structures (4). These molecules act as natural antibiotics against a wide variety of microorganisms (bacteria, fungi, parasites and virus) and they induce killing in a short contact time (5). Despite diversed sequence and secondary structures , the biological activity of AMPs have been characterized with sets of physicochemical traits and universal structural signatures (6).

The rising problem of antibiotic-resistance microorganisms to conventional antibiotics has prompted many researchers to develop AMPs as candidates of novel antibiotics (7-10). Microbial agents showed less efficiency in developing effective resistance mechanisms against AMPs than against classical antibiotics (11). Moreover, AMPs can act synergistically with classical antibiotics to improve their therapeutic activity (12). Large collections of AMPs both extracted from the natural sources and the engineered peptide variants have been documented to date and the number is expected to expand continuously in the near future.

Three major theories describing the killing of target microbes by AMPs have been postulated: 1) The loss of microbial viability might be due to the snowballing effects of energy exhaustion following equilibration of intracellular and extracellular ion concentrations through the disrupted membrane (10); 2) AMPs might create pores that admit water but do not allow osmotically active substances

to pass. The entry of water causes buildup of excessive osmotic pressure that eventually stretches and breaks the microbial membrane (7);3) AMPs could also penetrate the target cell through the disrupted membrane, bind to the intracellular molecules and disrupt their metabolic function (13). These unique mechanisms of action enable AMPs to avoid the common resistance mechanisms observed for conventional antibiotics (14). The mechanism of actions and selectivity of AMPs are suggested to be influenced by several physicochemical properties which includes charge net charge, hydrophobicity, and specific amino acid present in the peptide sequence. In this review, the influences of these three factors on the antimicrobial activity of AMPs will be discussed.

Net Charge

The most widely accepted model of antimicrobial by AMPs is the non-receptor mediated membrane lytic mechanism (15-16). The differences between prokaryotic and eukaryotic membranes enable selective targeting of AMPs against the microbes but not the host cells (17). In most cases, electrostatic interaction represents the main attraction force motivates the first contact between AMPs and microbes (4,18-20). All biological membranes are composed primarily of proteins and phospholipids. Unlike cell membranes of animals, microbial membranes are rich in anionic phospholipids, a characteristic property favored by AMPs. For instance, the presence of anionic teichoic acids (TAs) and LTAs in Gram-positive bacterial cell wall and lipopolysaccharides in Gram-negative bacterial cell wall attract the positively charged antimicrobial peptides to bind to the microbial cells membranes and make them preferred by AMPs over the mammalian cells membranes (17). On the contrary, the cell membranes of animals are rich in neutral phospholipids and cholesterol, substances that inhibit the integration of these peptides into membranes and the formation of pores. The difference in membrane composition between prokaryotic and eukaryotic cells represent the main contributor to AMPs cell selectivity against microbial pathogens while cytotoxicity against eukaryotic cells usually occur at higher concentrations of peptide (21).

Cationicity as the main factor describing the antimicrobial activity of AMPs have been documented in a number of studies (Table 1). Ringstad *et al*. found that the ability of peptides to disrupt microbial membranes increased with increasing net charge and hydrophobicity, and *vice versa* (22). Matsuzaki *et al*. studied the effect of net charge on the antimicrobial activity of Magainin 2, an AMP isolated from the amphibian *Xenopuslaevis*skin. Based on the parent peptide four analogs (MG0, MG2+, MG4+, and MG6+) have been designed with net charges ranging from 0 to +6. Peptides with higher positive charges were correlated with enhanced binding affinity against the negatively charged artificial membranes, suggesting that net charge

Table 1. *Antimicrobial activity of AMP analogues designed with variable net charge property.*

Peptide name	Peptide sequence	Net charge	Antimicrobial activity						Reference
			Radial diffusion assay at 100 μM (estimated to the nearest zone, mm)						
			P. aeruginosa		*B. subtilis*				
CNY21	CNYITELRRQHARASHLGLAR	+3	4.5		6.2				Ringstad *et al.*, 2007 (22)
CNY21L	CNYITELRRQLARASLLGLAR	+3	4.6		4.7				
CNY21K	CNYITELRRQKARASKLGLAR	+5	5.3		7.2				
CNY21R-S	CNYITELSSQHASASHLGLAS	-1	Not determined		Not determined				
MG0	GIGKFLHSAEEWGKAFVGEIMNS	0	No MIC data, based on artificial membrane analysis						Matsuzaki *et al.*, 1997 (23)
MG2+	GIGKFLHSAEKWGKAFVGEIMNS	+2							
MG4+	GIGKFLHSAKKWGKAFVGEIMNS	+4							
MG6+	GIGKFLHSAKKWGKAFVGQIMNSamide	+6							
			MIC (μM)						
			E. coli O111:B4	E. coli D21	E. coli O26:B6	A. baumannii	P. aeruginosa	S. aureus	
K5L7	KKLLKLLLKLLK-NH2	+6	25	33	25	50	37.5	37.5	Rosenfeld, Lev, & Shai, 2010 (24)
C6-K5L7	CH3(CH2)4CO-KKLLKLLLKLLK- NH2	+5	12.5	6.25	12.5	10	10	6.25	
C8-K5L7	CH3(CH2)6CO-KKLLKLLLKLLK- NH2	+5	6.25	4.5	6.25	4.5	3	3.12	
K7L5	KKLLKKLKKLLK- NH2	+8	50	5.	50	>100	>100	>100	
C6-K7L5	CH3(CH2)4CO-KKLLKKLKKLLK- NH2	+7	25	25	12.5	37.5	37.5	50	
C8-K7L5	CH3(CH2)6CO-KKLLKKLKKLLK- NH2	+7	12.5	10	6.25	19	15	20	
K9L3	KKKLKKLKKKLK- NH2	+10	50	50	50	>100	>100	>100	
C6-K9L3	CH3(CH2)4CO-KKKLKKLKKKLK- NH2	+9	50	50	50	100	100	100	
C8-K9L3	CH3(CH2)6CO-KKKLKKLKKKLK- NH2	+9	50	50	50	50	50	100	
			MIC (μg/ml)						
			P.aeruginosa	***E.coli***	***S.typhimurium***	***S.aureus***	***S.epidermidis***	***B.subtilus***	
+1	Ac–KWKEFLKEFKEAKKEVLHEALKAISEamide	+1	>64	32	>64	>64	64	32	Jiang *et al.*, 2009 (25)
+4E	AcKWKEFLKTFKEAKKEVLHTALKAISSamide	+4	8	8	4	>64	16	32	
V13K	AcKWKSFLKTFKSAKKTVLHTALKAISSamide	+7	4	8	4	32	8	32	
+8	AcKWKSFLKTFKSAKKKVLHTALKAISSamide	+8	8	8	4	>64	8	32	
+9	AcKWKSFLKTFKSAKKKVLHKALKAISSamide	+9	4	8	4	>64	8	16	

Abbreviation: MIC, minimum inhibitory concentration.

plays an important role in the binding process (23). In addition, Rosenfeld *et al*. demonstrated that increasing the peptide's net charge enhanced both antimicrobial and LPS neutralization activities of synthetic AMPs (24). Moreover, Jiang *et al*. showed that net charge has an imperative effect on the antimicrobial activity of L-V13K peptides (25). Decreasing the net charge of L-V13K analogs to levels below +4 reduce antimicrobial and hemolytic activity, and increasing the net charge from +4 to +8 can increase the antimicrobial activity (25). However, increasing the net charge to above +9 improved the antimicrobial activity but dramatically increased the undesired hemolytic activity (25).

Although cationicity of AMPs can be elevated by inserting/substituting the native residues with positively charged amino acids, this can also be achieved by reducing the proportion of negatively charged residues. Ueno *et al*. designed the three synthetic analogues NP1P, NP2P, and NP3P by using the acid-amide substitution approach, which replaced the acidic Asp and Glu to the neutral amidated residues (Asn, Gln) (26). This method prevents the potential dramatic structural changes to the peptides following substitution of unrelated amino acids. A gain in cationicity was thus achieved with indirect reduction of peptide's total negative charge. Interestingly, the newly generated peptides displayed substantial increase in antibacterial activity against both Gram-positive and Gram-negative bacteria *Bacillus subtilis*, *S. aureus*, *S. typhimurium*, *P. aeruginosa, E. coli*, and *Serratia marcescens*.

Hydrophobicity

Due to the presence of non-polar amino acid residues in the sequence, AMPs are commonly characterized by the hydrophobicid properties. Hydrophobicity rules the ability of AMPs to partition into the lipid bilayer of microbial membrane. However, increasing levels of hydrophobicity are strongly associated with mammalian cell toxicity and loss of antimicrobial specificity (17). Thus, careful consideration is necessary when altering the peptide hydrophobicity.

To investigate hydrophobicity as a function of antimicrobial activity, Wieprecht *et al*. generated a series of magainins analogues to which charge, helicity, and hydrophobic moment parameters were kept invariable while only the hydrophobicity of the peptides was systematically altered (27) (Table 2). They revealed that increase the peptide hydrophobicity can enhance antimicrobial activity although it was also correlated with hemolytic activity. Furthermore, peptides differing in hydrophobicity were found to display different spectrum of antibacterial activity (27). As for *P. aeruginosa*, the specificity of this class of

Table 2. *Antimicrobial activity of AMP analogues designed with variable hydrophobicity.*

Peptide name	Peptide sequence	Hydrophobicity[a]	Antimicrobial activity			Reference
			E. coli	*P. aeruginosa*		
$L^2R^{11}A^{20}$-M2a	GLGKFLHSAKRFGKAFVGEAMNS	-0.091	75	>75		Wieprecht *et al.*, 1997 (27)
M2a	GIGKFLHSAKKFGKAFVGEIMNS	-0.091	38	76		
I^6L^{15}-M2a	GIGKFIHSAKKFGKLFVGEIMNS	0.200	19-38	76		
$I^6A^8L^{15}I^{17}$-M2a	GIGKFIHAAKKFGKLFIGEIMNS	0.326	2.4	19-38		
			MIC (μM)			
			P. aeruginosa			
6K-F17	KKKKKKAAFAAWAAFAA-NH2	-0.253	4			Yin *et al.*, 2012 (29)
6K-F17-4L	KKKKKKALFALWLAFLA-NH2	0.218	16			
3K-F17-3K	KKKAAFAAWAAFAAKKK-NH2	-0.253	32			
3K-F17-4L-3K	KKKALFALWLAFLAKKK-NH2	0.218	8			
			MIC (μg/ml)			
			S. aureus	B. subtilis	E. coli	
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2	0.273	0.72	0.18	11.4	Yan *et al.*, 2003 (30)
Mel(12-26)	GLPALISWIKRKRQQ-NH2	-0.607	5.6	5.6	88.9	
Mel(12-26, L^1)	LLPALISWIKRKRQQ-NH2	-0.327	1.1	2.2	70.8	
Mel(12-26, S^2)	GSPALISWIKRKRQQ-NH2	-0.913	90	67.5	>270	
Mel(12-26, L^3)	GLLALISWIKRKRQQ-NH2	-0.247	1.2	2.2	70.8	
Mel(12-26, S^3)	GLSALISWIKRKRQQ-NH2	-0.553	3.1	1.6	6.25	
Mel(12-26, L^4)	GLPLLISWIKRKRQQ-NH2	-0.473	1.3	2.4	76.7	
Mel(12-26, S^5)	GLPASISWIKRKRQQ-NH2	-0.913	250	31.2	>250	
Mel(12-26, S^6)	GLPALSSWIKRKRQQ-NH2	-0.960	14.6	39	311	
Mel(12-26, L^7)	GLPALILWIKRKRQQ-NH2	-0.300	1.4	0.64	81.7	
Mel(12-26, L^8)	GLPALISLIKRKRQQ-NH2	-0.293	13.5	10.0	161	
Mel(12-26, S^8)	GLPALISSIKRKRQQ-NH2	-0.600	38.2	76.4	>305	
Mel(12-26, S^9)	GLPALISWSKRKRQQ-NH2	-0.960	94.2	70.8	>94.4	

[a]Calculated based on Grand Average of Hydrophobicity (GRAVY) using ExPASyProtparam tool (http://web.expasy.org/protparam/).
Abbreviation: MIC, minimum inhibitory concentration.

magainins analogues appeared to reduce with increasing hydrophobicity, but this was not the case for *E. coli*.

Chen *et al*. investigated the role of hydrophobicity in the antimicrobial activity of the α-helical AMPs by systematically decreasing or increasing the hydrophobicity of a synthetic V13KL peptide (28). They noted that, decreasing peptides' hydrophobicity was associated with reduced antimicrobial activity. Improving the antimicrobial activity of the peptides by enhancing the hydrophobicity was achievable, however, only up to a certain level. Deviation from the specific hydrophobicity window would result in the significant loss of antimicrobial activity with the peptides (28). This occurs most likely due to increased peptide dimerization, which prevent the access of peptide monomers on microbial cell membranes. The lower hydrophobic variant 6K-F17 designed by Yin *et al*. was found to possess a four-fold higher antipseudomonal activity as compared to the Ala-substituted analogue 6K-F17-4L (29). Furthermore, 6K-F17 displayed minimal hemolytic activity up to the concentration of 320 μM as compared to 6K-F17-4L which showed 40 – 80% hemolysis at the same level. In addition, their data suggested that peptides with higher hydrophobicity have stronger self-association and aggregation tendencies than those with lower hydrophobicity level (29).

Yan *et al*. extracted 15 residues from the C-terminal segment of melittin and systematically altered the hydrophobicity of the peptides via individual residual substitution. The investigators found that increased in hydrophobicity but not amphipathicity enhanced antimicrobial activity (30). Moreover, they noticed that the effect of changing the individual residues was stronger at specific positions in the sequence (30). Interestingly, the antimicrobial and hemolytic activities were suggested to favor the opposite faces and this indicates that antimicrobial activity could be dissociated from the side effects with careful consideration on region/face of the peptides (30). Cornut *et al*. found that membrane lysis increased with increasing of peptide hydrophobicity (31). Notably, our preliminary results also underlined the antipneumococcal-enhancing effect by increasing the hydrophobicity of a series of peptides with fixed sequence length. We also emphasized the importance of optimum hydrophobicity window to which higher or lower hydrophobicity levels would impair the antimicrobial activity of the peptides. Hydrophobicity has also been found to be a key parameter governing the antimicrobial activity of AMPs in a number of quantitative structure–activity-relationship investigations. However, this physicochemical property shall be manipulated carefully to avoid increase of the unwanted hydrophobicity-associated peptides' toxicity on human cells(32).

Presence of specific amino acids residues

Many antimicrobial peptides encompass an unusual composition of amino acids. One group of particular interest is peptides with high content of Arginine (Arg) and Tryptophan (Trp) (33) (Table 3). Beside their importance in antimicrobial peptides, Trp play a crucial role in membrane spanning proteins, as Trp has a strong preference for the interfacial regions of lipid bilayers(34). Additionally, antimicrobial peptides with high content of Arg and Trp were found to possess the highest antimicrobial activities (35).The Arg side chain is capable of forming almost as many hydrogen bonds with the surrounding water molecules as when it is not involved in any cation–π interactions. This is in contrast to Lysine (Lys), which cannot form hydrogen bonds while engaged in cation–π interactions with an aromatic residue (36). This difference is responsible for the increased activity of Arg containing peptides over Lys substituted peptides. Many studies well documented that Trp residues have a preference for the interfacial region of lipid bilayers.

Lawyer and his fellow workers (37) tested the antimicrobial activity of 13 amino acid Trp-rich peptide and found that the peptide has strong antimicrobial activities against various Gram-positive and negative bacteria and fungi. In addition, Torcato *et al*. (12) studied the effect of Trp and Arg on the antimicrobial activity of peptides against Gram-positive and Gram-negative bacteria. In their study, they designed two new peptides RW-BP100 and R-BP100 based on previously designed peptide BP100 by the same group of researchers. In these two analogues they replaced Tyrosine (Tyr) with Trp, and Lys with Arg. Their results showed that the new peptides have stronger activity against both Gram-negative and Gram-positive bacteria. In general, the cationic nature and unique hydrogen bonding geometry of Arg and the complex properties of Trp enhance the ability of AMPs to interact and destroy the microbial membrane. The positive charge of Arg effectively increases attracting ability of the peptide to the target membranes, and hydrogen bonding assists its interaction with negatively charged surfaces. Trp represents the most suitable amino acid to enable the peptide to associate with the target membrane (33).

Functional group alteration also represents a potential strategy to enhance the antibacterial potency of peptides and to increase the proteolytic resistibility of the peptides (38). The strong antibacterial and fungicidal activities of Thanatin can be attributed to the four specific peptide regions: the C-terminal loop, the three residue extension at C-terminus, presence of seven hydrophobic residues at the N-terminus, and three N-terminal residues which is indispensable for antifungal activity (39). A C-terminally amidated thanatin which is an insect-derived *Podisusmaculiventris* AMP showed strong antibacterial effect against Gram-negative bacteria, in particular, the extended-spectrum B-lactamase-producing *E. coli*. (38-39). This peptide also conferred dose-dependent therapeutic efficacy with survival rates of 50.0%, 66.7%, 91.7% following low-, medium-, and high-dose A-thanatin treatment in a mice septicaemic model as compared to 0% survival at day 2 post treatment using ampicillin (38).

Table 3. *AMP analogues designed to investigate the role ofspecific amino acids in relation to antimicrobial activity.*

Peptide name	Peptide sequence	Antimicrobial activity								Reference
		MIC (μM)								Torcato *et al.*, 2013 (12)
		E. coli	*K. pneumoniae*	*P. aeruginosa*		*S. aureus*	*S. pneumoniae*	*E. faecium*		
RW-BP100	RRLFRRILRWL-NH2	0.2±0.1	0.3±0.0	2.5±0.5		0.3±0.1	0.4±0.1	1.0±0.0		
R-BP100	RRLFRRILRYL-NH2	0.4±0.1	0.6±0.3	2.0±0.0		3.0±0.6	4.0±0.0	2.0±0.0		
BP100	KKLFKKILKYL-NH2	0.7±0.2	4.0±0.0	8.0±0.0		16.0±0	16.0±0.0	16.0±0.0		
		Aspergillusfumigatus			*Candida albicans*					Lawyer *et al.*, 1996 (37)
1	VRRFPWWWPFLRR	250			1000					
2	RRRFPWVCWPFLRRR	250			1000					
3	RFPWWWPFLR	500			>1000					
4	FPWWWPF	500			>1000					
		MIC (μg/ml)								Hou *et al.*, 2011 (38)
		S. aureus	*E. coli* ATCC25922	*E. coli* ATCC35218	*E.coli* HN10349	*E.coli* HN10318	*E.coli* SX49660	*E.coli* XJ74283	*E.coli* JT11092	*K. pneumoniae*
A-thanatin	GSKKPVPIIYCNRRTGKCQRM	256	16	4	2	2	4	8	2	4

Abbreviation: MIC, minimum inhibitory concentration.

Discussion

In the face of increasingly common antibiotic-resistant microbes, novel antimicrobial agents are urgently needed to serve as standalone therapeutics or in combination to support the use of conventional antibiotics. AMPs possess multiple advantages in term of its potent and rapid bactericidal activity while also being broad spectrum to serve as the candidate for alternative drug development. The design of new synthetic analogues of AMPs mainly focus on one common goal – better therapeutic index with higher antimicrobial activity and lower cell toxicity levels. This will require careful and systematic design strategy. The physicochemical properties of AMPs represent important factors to be considered as net charge, hydrophobicity, and the specific amino acids presence would affect the antimicrobial activity of the peptides. More studies have been documented in support of the notion that increasing net charge (to a certain limit) shall be followed. The same principle goes to the hydrophobicity of the peptides. Moreover, certain amino acids are responsible for the antimicrobial activity but not cytotoxicity of the peptides and vice versa. Although there are many factors to be considered in designing AMPs, one should not interpret these factors independently, but to consider all factors within the same formulation.

Although thousands of peptides have been discovered and synthesized in the past decades, only limited fraction of them have been studied and tested for potential development. Several AMPs possess excellent antimicrobial activity *in vitro*. However, like any new class of drugs, AMPs need to gain approval through multiple clinical assessments and trials before being brought into the actual clinical uses by the pharmaceutical companies. These challenges include drug stability *in vivo*, associated toxicity to human cells, demonstration of good antimicrobial activity, and the relatively high costs in peptide-antibiotic development and manufacturing.

Up to date, only limited success has been achieved with those AMPs that have entered into clinical trials and to the best of our knowledge, none have obtained FDA agreement for clinical use. Despite the fact that AMPs are essential components of the host innate immune system against microbial pathogens, their possibilities as a new class of therapeutic agents still remain to be proven. Nevertheless, the eventual goal to develop AMPs into clinically useful drug should not have just been hampered by the obstacles and further exploration in the field should continue.

Acknowledgement

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AUTOLOGOUS CHONDROCYTE IMPLANTATION FOR KNEE FOCAL CARTILAGE DEFECTS: 3 YEARS' FOLLOW-UP AT THE UNIVERSITY MALAYA MEDICAL CENTRE

Abbas AA[1], Mohamad JA[2], Lydia AL[3], Selvaratnam L[4], Razif A[1], Ab-Rahim S[1],Kavitha G[1], Shilpa PN[5], Kamarul T[1]

1 *Tissue Engineering Group, NOCERAL, Department of Orthopaedic Surgery, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia*
2 *Selangor Medical Centre, Shah Alam, Selangor, Malaysia*
3 *Department of Rehabilitation Medicine, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia*
4 *School of Medicine and Health Sciences, Monash University Malaysia, Petaling Jaya, Selangor, Malaysia*
5 *Virtis Bio Labs, Salem, India*

Correspondence:
Dr. P. N. Shilpa
Virtis Bio labs
39, Eswari Gardens Annex,
Mannarpalayam
Kannankuruchi, Salem – 636 008
Telephone : +91-95 97 92 68 27
E-mail : shillpamail@gmail.com

ABSTRACT

Autologous chondrocyte implantation (ACI) is a widely accepted procedure for the treatment of large, full-thickness chondral defects involving various joints, but its use in developing countries is limited because of high cost and failure rates due to limited resources and support systems. Five patients (age <45 years) with focal cartilage defects received ACI at University of Malaya from 2006 to 2007 and followed up for 36 months. The average presubjective Knee Evaluation Forms (IKDC) improved from 38.44±6.29 to 25.6±8.04 postoperatively, the Oxford Knee Score (OKS) went from 25.6±8.04 to 13.96±1.63 and the American Knee Society Score (AKSS) improved from 80±14.33 to 92.96±5.82 post-operatively. Thus improvements were seen in the IKDC and AKSS score but not in the OKS. Magnetic resonance images showed the presence of cartilage tissue filling in the lateral and medial patellar facet and medial femoral condyle in three patients. Failures were seen in two patients, both with patellar defects and over the age of 36 years. Treatment with autologous chondrocyte implantation for focal cartilage defect in lateral and medial patellar facet and medial femoral condyle showed early improvement which was maintained at 3 yrs follow-up. ACI provided satisfactory outcome in focal cartilage defects involving the femoral condyle.

Keywords: *Knee, cartilage defect, autologous chondrocyte implantation, chondrocyte*

Introduction

Focal chondral lesions pose a challenging problem to treat owing to loss of blood supply, low mitotic activity and immobility of articular chondrocyte (1). Although a number of surgical options are available to treat this increasingly common condition, each method has its own advantages and disadvantages (2). The aim of all cartilage repair methods is to reestablish functional properties of the damaged chondro-osseus unit. Debridement (3), drilling (4), and microfracturing of subchondral bone (5) have been advocated as treatment modalities. More recently, autogenous osteochondral plugs, osteochondral allografts, and autologous chondrocyte implantation (ACI) have been used. These techniques have been reported to have good short- to mid-term results (6).

ACI is a two-stage procedure involving the harvesting of cartilage from a non-weight-bearing area of the joint, followed by chondrocyte isolation and culture. Cells are then harvested from culture and reimplanted into defective sites. These cells undergo further proliferation and de-differentiation within the damaged area (7). Newly formed tissue within the treated area will mature over time, developing into more organized tissue that mimics the surrounding native cartilage (hyaline-like cartilage) (8). Because of the novelty of this treatment, ACI continues to be evaluated for its consistency and reproducibility in

producing good clinical outcomes (8-11). The durability of repair and cost effectiveness is also being questioned (12). Despite the popularity of the technique, the factors influencing the functional outcome after ACI are still poorly understood. Furthermore, studies using ACI in developing countries that have restricted resources are limited. This report describes the results of a preliminary cohort study conducted at the University of Malaya to determine the clinical outcome of five patients aged below 45 years who were treated for focal cartilage defect using ACI.

Materials and methods

Patients

Five patients aged below 45 years underwent ACI for treatment of focal knee cartilage defects between 2006 and 2007. Approval to conduct the study was given by the medical ethics committee (Medical Ethics Committee Ref No: 553.37), University of Malaya. In accordance with Malaysian law, patients were informed of the nature of the study and provided written consent. Patients' histories, detailing the mechanism of injury, onset, and symptoms as well as prior treatments were recorded for preoperative assessment. Patients with cartilage defects down to, but not through, the subchondral bone, on a load-bearing surface of the femoral condyle or the patellar facet were recruited for treatment. The patients presented with a variety of complaints, including localized pain, swelling, and retropatellar crepitus. Preoperative assessments included use of the 2000 IKDC Subjective Knee Evaluation Forms (13), the Oxford Knee Score (OKS) (14), the American Knee Society Score (AKSS) (15), anteroposterior and lateral knee radiographs and magnetic resonance imaging (MRI) .The ACI technique was performed as described by Brittberg *et al*. (16).

Isolation and Culture of Chondrocytes

Patients underwent diagnostic arthroscopy of the knee under general anaesthesia. A tourniquet-controlled, bloodless field provided good visualization of the defect(s) sites as well as the areas suitable for cartilage harvesting. Approximately 2-3 g of cartilage tissue was removed using a surgical punch. The harvested tissue was placed in a sterile container containing phosphate-buffered saline supplemented with penicillin and streptomycin. The tissue samples were sent to the laboratory for further processing within 4 hours of retrieval.

Chondrocytes were cultured in an ISO-certified class 1000 clean laboratory located in the Faculty of Medicine, University of Malaya. Harvested tissue was minced before being digested in collagenase-type II solution for 24 h. The following day, the suspension was centrifuged at 1800 rpm for 10 min to produce the cell pellet. The pellet was resuspended in DMEM/F-12 growth medium at a ratio of 1:1, supplemented with 10% fetal bovine serum and 25 µg/ml of ascorbic acid. Cultures were stored in 5% CO_2 and 98% humidity at 37°C. The medium was replaced every 3-4 days. Observation continued until 80% cell confluence was reached, after which the culture was trypsinized to detach the cells from the plastic surfaces for further passage. Cell cultures were expanded up to the third passage to allow the recovery of 2-5 x 10^6 cells, which took approximately 4–6 weeks. Cell viability was analysed using trypan blue technique (17).

Implantation

Prophylactic antibiotics (Cefuroxime sodium, 750 mg) were given intravenously in three doses over 24 hours during and after surgery. Cell implantation was performed as described by Brittberg *et al*. (16). A medial or lateral parapatellar arthrotomy was performed in a tourniquet-controlled, bloodless field. To ensure that only healthy tissue remained in the defect, the margin of the defect was excised whilst the base was scraped using a scalpel to remove tissue remnant. Care was taken to ensure that the subchondral plate was not penetrated. A periosteal flap, identical in shape and size with the lesion area, was harvested from the medial aspect of the proximal tibia, or the supracondylar region of the femur of the affected knee. This flap was used to cover the cartilage defect, with the cambium layer facing the subchondral bone in the defect area. The flap was sutured to the surrounding rim of the normal cartilage with interrupted 6-0 Vicryl® sutures, leaving an opening in the upper part of the defect for insertion of cultured chondrocytes. The intervals between the sutures were sealed with fibrin glue, and the patch was tested for water-tightness by injecting saline into the defect and checking for leakage. More than 48×10^6 cultured chondrocytes (four vials) were administered beneath the periosteal flap, and the opening was closed with suture and fibrin glue. The joint capsule, retinaculum layer and skin were sutured in separate layers. In the case of patellar maltracking, the lateral parapatellar joint capsule was not repaired. The knee was covered with a small elastic bandage.

Postoperative Protocols

Continuous passive motion was initiated within 6 hours postoperatively, with the range of flexion limited to 30°. This was continued until patients were able to mobilize the knee independently. Quadriceps strengthening exercises were encouraged during the recovery period. Active movement of the knee without weight-bearing was initiated 2–3 days after surgery. Patients were discharged with a protective knee brace that limited flexion to 45°. Once discharged, patients attended outpatient physiotherapy twice weekly, initially for 12 weeks and then, subject to their progress, once weekly. Weight-bearing was gradually increase, along with knee flexion increased to full extension, with isometric quadriceps training during the first 8 weeks after surgery.

Follow-up

Patients were evaluated at 3, 6, 12, 24 and 36 months postoperatively. Evaluation with the 2000 IKDC Subjective Knee Evaluation Forms, OKS and the AKSS was performed at 3-monthly intervals, while MRI assessments were performed at 6 months postoperatively. The AKSS score included surgeons' reports of patients' symptoms, impairment (clinical examination findings) and disability measured with the AKSS before and 1 year after surgery. The AKSS generates a knee score from 0 to 100, based on symptoms and impairment, and a function score from 0 to 100 calculated from the answers to questions on disability. Higher scores indicate lesser symptom severity, impairment or disability. The OKS, a joint-specific instrument, consists of 12 questions to assess pain and physical disability on a five-point Likert scale (1 representing no pain or disability, to 5 representing extreme pain or disability), which yields a single score (i.e. sum of all individual items) ranging from a best functional outcome of 12 to a worst functional outcome of 60.

Results

The five patients (two men, three women) were aged 25–45 years (mean, 37.2 years). The average defect size was 8 cm^2 (range: 4.0-12.0 cm^2). Demographic data for all five patients who underwent ACI are shown in **Table 1**.

The IKDC, OKS and AKSS scores increased in three patients after ACI as early as 3 months postoperatively and continued to improve up to 24 months and remained at the same level up to 36 months. The mean age of patients who responded well to ACI was 36years, while that of patients who had a poorer outcome was 39.5years. The average preoperative and postoperative IKDC, OKS and AKSS scores of patient are shown in **Table 2**.

It can be argued that while the high scores at 3 months may be controversial since patients are not fully ambulating, the data presented here is outcome perceived by the patients and not really of the objective physical outcome. There was little or no pain and satisfaction was high in all patients at 3 months. This may have explained the reason for the high score observed at 3 months follow-up.

At 36 months' follow-up, three patients had excellent results, while in two patients knee function was found to be poor, as reflected in decreased IKDC, OKS and AKSS scores (**Figures 1, 2, 3**). MRI of three patients with good knee functional scores showed that the defective area was completely filled with reparative tissue, highlighted with hyperintense signals. In the two patients with a poor clinical response, the area surrounding the repaired cartilage had lower density. In the three patients with good clinical response i.e. OKSS of less than 15 and IKDC of more than 50, an increase of 2-3 mm in the depth of the repair tissue was observed over time, being most remarkable 24-36 months after surgery (**Figure 4).**

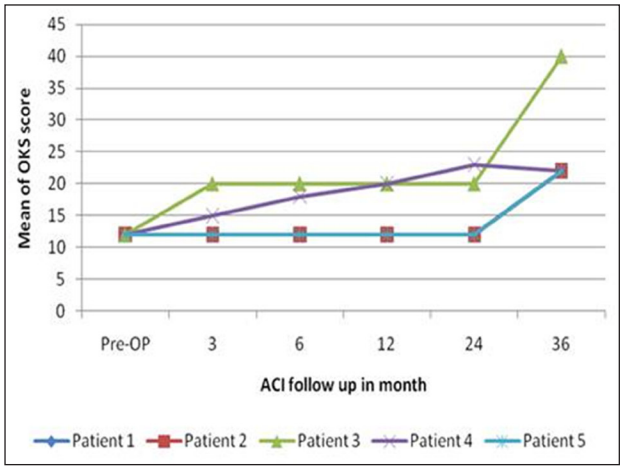

Figure 1: *Mean AKSS score preoperatively and after 3 years' follow-up. Patients 1 and 2 have same mean score.*

Table 1: *Demographic data of patient receiving ACI to treat knee focal cartilage defect*

Case	Gender	Age	Cartilage lesion		Mechanism of Injury	Outcome
			Site	Size (mm)		
1.	Male	45	Lateral and medial patellar facet	8	Patellar maltracking	Successful
2.	Female	34	Medial femoral condyle	4	Sports	Successful
3.	Male	29	Medial femoral condyle	4	Sports	Successful
4.	Female	33	Lateral patellar facet	4	Patellar maltracking	Failure
5.	Female	45	Trochlea and patella	4	Trauma	Failure

Table 2: *Outcome of OKS, IKDC, AKSS scoring of patient receiving ACI to treat knee focal cartilage defect during three years follow-up.*

Score	Preoperatively	3 months	6 months	12 months	24 months	36 months
OKS	25.6 ± 8.0	15.8 ± 5.3	15.2 ± 4.3	14.8 ± 3.8	12 ± 3.4	14.2 ± 3.12
IKDC	38.44 ± 6.2	62.4 ± 18.4	64.8 ± 20.0	67.4 ± 22.4	83.3 ± 23.5	83.35 ± 5.7
AKSS	80 ± 14.3	86.6 ± 16.4	88.6 ± 15.6	89.6 ± 14.2	100 ±14.2	100 ± 12.7

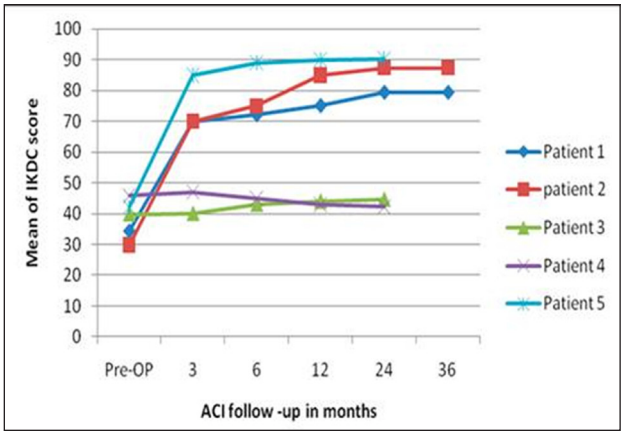

Figure 2: *Mean IKDC score preoperatively and after 3 years' follow-up.*

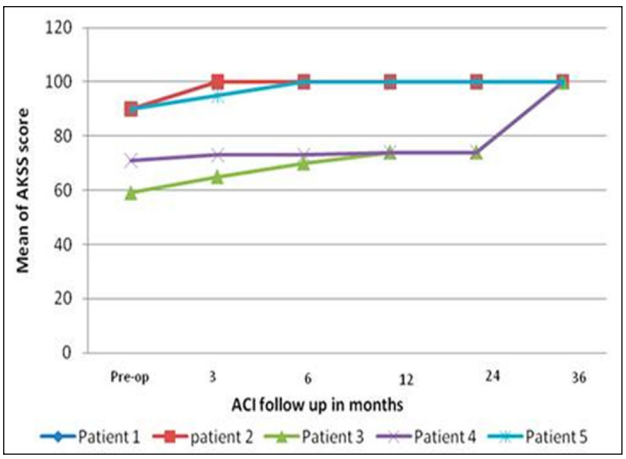

Figure 3: *Mean OKS score preoperatively and three years' follow-up. Patient 1, 2 and 5 have same mean score*

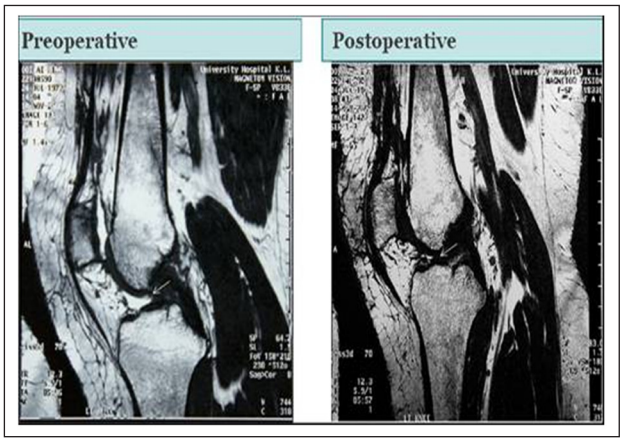

Figure 4: *Magnetic resonance images obtained 3 year after autologous chondrocyte implantation preoperatively and postoperatively*

Discussion

An overall improvement in patient functional scores for up to 3 years has been observed in patients who underwent ACI. This preliminary study on the use of ACI in selected patients reveals that this technique can be used successfully. There appears to be a dramatic improvement in patient satisfaction and knee function within 3 months following surgery (38-60%) and continue to increase over time. This finding was similar to that reported by Micheli *et al*. which demonstrated an average improvement of up to 84% in patients who underwent ACI after 2-3 years (10).

The major limitation of this report is the small number patients. However, this report does provide preliminary evidence advocating its use for continued recruitment for patients in a large scale study. The results also suggest that patients with a patellar defect: may not be suitable candidates. Similar outcomes were also observed in previous published results (8).

Although in a number of studies, it has been reported that low number of cells implanted in cartilage defects may have contributed to the failures observed, however it is unlikely to be the case within the present study. In patients recruited for this study, at least 48 $\times 10^6$ cells (4 vials) were administered to each patient, which was similar if not higher than those reported in previous studies (18). Although the optimal number of required cells has not yet been determined, high cell densities seem to be desirable and recommended by most authors (19). In several studies, the number of chondrocytes seeded in the initial defects appears to be linearly correlated to the biosynthetic activity required for cartilage restoration (20). The higher numbers of cells used in this study as compared to those used in conventional studies suggests that the use of higher number of cells would not have influenced the outcome. Instead, the site of injury may be the determinant factor which may influence the outcome of the repair process.

As with previous reports, patient age has been found to be a confounding factor for the success in ACI (11). In our study, patients under 36 years showed better improvement in all scores than those exceeding 36 years. This may have resulted from the use of ageing chondrocytes, related to the advanced age of the donors. It has been suggested that the ageing chondrocytes have limited regenerative ability by producing limited extracellular matrix hence, resulting in poor repair outcomes (5). Nevertheless, even in patients with degenerative cartilage lesions, the use of ACI may be feasible albeit with limited success (21-23). However, this issue remains controversial and requires substantial evidence before it can be routinely advocated.

Of the many available methods used to access cartilage restoration, many studies have advocated the use of MRI, mainly because it is non invasive, reliable and, allows comparative analyses to be made between the pre- and post-operative conditions (24). In patients who were successful at 3 years, 2-3 mm filling of what appears to be cartilage tissue i.e. not bone and not synovial due to the apparent density, within the medial femoral condyle and patella defects were observed. It is not unexpected that patients with patellofemoral defects are more likely

to fail, as lesions of the patellofemoral joint have always been more demanding and more difficult to treat than femoral condyle lesions (25). This can be explained by the more complex pathogenesis involved resulting from the complicated anatomical malfunction which causes patellofemoral malalignment, maltracking and ultimately the instability of the patella. In a report published by Peterson *et al*. (26) involving 94 patients who underwent ACI and had been followed up for 2–9 years, good or excellent clinical outcomes were achieved in 76% of the patients with isolated condylar lesions while the worst outcome was seen in those who had multiple defects or trochlear lesions and patella. Peterson *et al*. (9) also showed that lesions of patellar femoral joint were more difficult to treat than femoral condyle lesions.

Based on available literature, it appears that the repair process following implantation of autologous chondrocytes may be the result of multiple biological and mechanistic cellular functions acting in concert (27). It has been suggested that among those most commonly described, the repopulation of implanted chondrocytes within the defect sites appears to be the more accepted notion (28). Once adapted into the surrounding environment, these cells are said to produce extracellular matrix which inherently provides the repair tissue which is observed at the end of the repair process. There is however, no clear evidence to support this assumption as studies have demonstrated that these implanted cells do not proliferate when contained within the defect site and up to 87% of these cells undergo apoptosis within 4 weeks (29-31). It has also been proposed that it is not the transplanted cells which result in the repair but the use of periosteum as a cover which promotes tissue healing (32). However, this too has been refuted as studies involving rabbits have shown that using periosteum alone does not result in repair of the cartilage tissues (32-35). In addition, studies using synthetic patches instead of periosteum in patients also resulted in the regeneration of damaged cartilage (36).

While it is not clear as to what causes the cartilage defect to undergo repair when ACI is used, our result showed apparent improvement in tissue repair as observed in many clinical and laboratory studies (36,37). Further studies to elucidate the causes and mechanisms leading to the regenerative process should therefore be conducted in larger and more robust experiments, with hopes that better understanding of ACI can be achieved, therefore producing superior results in clinical use.

Conclusion

The present case study provides support to use ACI in selected patients with focal cartilage defects involving the femoral condyle. The use of ACI in patients above the age of 36 or those with patellar defects are not encouraged, but need to be supported by larger scale studies.

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LOW ORGAN DONATION RATE IN MALAYSIA: A SURVEY

Makmor T[1], Abdillah N[2], Raja Noriza RA[1], Nurulhuda MS[3], Sook-Lu Y[3], Soo-Kun L[4], Kok-Peng N[4]

1 *Department of Administrative Studies and Politics, Faculty of Economics and Administration, University of Malaya, Kuala Lumpur*
2 *Tun Abdul Razak School of Government, Universiti Tun Abdul Razak, Kuala Lumpur*
3 *Department of Economics, Faculty of Economics and Administration, University of Malaya, Kuala Lumpur*
4 *Department of Medicine, Faculty of Medicine Building, University of Malaya, Kuala Lumpur*

Correspondence:
Makmor Tumin
Department of Administrative Studies and Politics, Faculty of Economics and Administration, University of Malaya, 50603 Kuala Lumpur.
E-mail: makmor@um.edu.my

ABSTRACT

Organ shortage is a major concern in many countries. The objective of this paper was to investigate the factors that contribute to the low quantity of organ donation in Malaysia. The 1311 respondents in this survey came from the three main ethnic groups in Malaysia (Malay, Chinese and Indian). The survey was based on these components: The reason for not pledging to become a donor; the reason of refusing to become a donor; and whether non-fungible incentive would influence decision. The lack of information and trust were the factors that influenced the respondents to remain apathetic to organ donation. The results denote that people are unlikely to become a donor even if non-fungible incentive were provided to them. Thus, it is important for the government bodies to evaluate the programme and strategies of public education in relation to organ donation.

Keywords: *Malaysia, organ donation, organ donation rate, organ shortage, public education*

Introduction

World over, the demand for organs far outweigh supply and Malaysia has one of the lowest deceased organ donation rates (0.48 donations per million populations in 2010) in the world. A similar scenario can be seen in countries like Myanmar (0.02), Guatemala (0.52), Bulgaria (1.14) and Thailand (1.26) (1).

One of the suggestions to overcome the problem of organ shortfall is for countries to implement the opt-out consent policy (2, 3, 4). Countries that follow the opt-out consent policy such as Spain (34.13), Belgium (25.61), France (25.31) and Austria (20.72) (1) have all experienced better rates of organ donation. While it is important to consider the opt-out consent policy, there are other policies that attempt to encourage organ donation. The range include: introducing paired organ donation drive where families of both the deceased donor and living donor are rewarded; promoting altruistic approaches by advocating the values of good Samaritan advocacy; implementing appropriate educational programs for the public and hospital staff regarding the need and benefits of organ donation; and managing the financial payment for the donor (5).

Some studies in Malaysia suggest the possibility of religio-cultural factor as the main impediment to organ donation in Malaysia (6, 7), while others suggest low organ donation – especially among the post-secondary education group – as the result of the public lack of trust toward the medical system (8). In another study, there was a proposal that non-fungible incentives would positively influence the way people react to organ donation (9). To facilitate organ donation, Malaysia introduced the Human Tissue Act in 1974, followed by a National Transplantation Program in 1975. The first living kidney transplant was performed in Malaysia in 1975 and the first deceased donation follow suit two years later.

Much later in 2007, Malaysia strengthened its existing transplantation policy by introducing the National Organ, Tissue and Cell Transplantation Policy. More recently, in 2011, the Government came out with a guideline and procedures to embark on 'unrelated living donation.' The effort though has given little effect. Up to December 2011, there were only 1122 kidney transplantation involving living donors, while only 378 transplantation involving deceased donors were recorded as of 31 July 2012 (10).

This paper is an attempt to explain the reasons that impede Malaysians to become donors by broadening our analysis to cover Malaysia's population on their willingness to become donor.

Methods

A survey to gauge public opinion on becoming deceased donor was carried out in the Klang Valley (Kuala Lumpur and its suburbs) between October and December 2010. Klang Valley was chosen because of its mixed ethnic population that mirrors Malaysia's demographic profile. The survey's ethnic breakdown also mirrors Malaysia's and Klang Valley's demographic profile: 58.1% Malays, 26.8% Chinese, 11.5% Indians and 3.6% others.

In the survey, 1420 people were approached, out of which 1311 agreed to do the survey, giving a response rate of around 91.5%. The enumerators were asked to approach people at three designated spots – university campuses, hospitals and eateries. The three locations were chosen due to the need of selecting area where diverse demographic characteristics of respondents can be found. University campuses for example, are the most possible location to gauge young peoples' views on organ donation. Hospitals are captive ground for potential organ donors and those with strong views on health issues, while eateries provide a ground for a wider spectrum of respondents. The enumerators were also asked to adhere to Malaysia's ethnic composition when approaching potential respondents. They were instructed to cease approaching people of an ethnic group when the quota for that particular group has been fulfilled.

After filling-up their background information in the questionnaire, the respondents were presented with a specific question, "Are you willing to donate your organ upon death?" This question is important to separate respondents into two clear-cut categories for further investigation: those who are willing and those who are unwilling to donate. In the following part of the questionnaire, we asked those willing donors on their reason for not registering as deceased donor despite saying "yes" to donation. Five options were given to them and they were allowed to choose more than one options. The options were: (i) I do not know the procedures on how to register; (ii) Still waiting for my family's consent; (iii) No motivation whatsoever to do so; (iv) Due to time constraint and, (v) Others.

The last two parts of the questionnaire were directed to the group who were unwilling to become deceased donor. We began to investigate the non-willing donors group by examining the reasons for the unwillingness. They were given two set of options. The first set of options was based on religio-cultural considerations: "It is against my religion" and "I want my body to remain intact after death." The second set was based on structural considerations: "I am not convinced that my organ/body part will be used beneficially" and "I do not have access to the information."

Next, we investigated the group who were unwilling donors on the possibility of them changing their mind. There were given two options: one, which is based on the importance of public education and the other, a policy driven by monetary incentive. The first question asked was, "Are you going to register as a deceased organ donor if you and your family were provided with sufficient information on the entire process of donation: from registration to the point of donation?" The second question was, "Are you going to register as a deceased organ donor if special benefits such as tax rebates were provided to registered donors?" This information will help in establishing people's preferences: whether they are more responsive to proper awareness campaigns or instead, more responsive to non-fungible incentive-driven policy.

Results

The breakdown of the respondents by ethnic group from the survey are as follows: 762 Malays (58.1%), 351 Chinese (26.8%), 151 Indians (11.5%) and 47 'Others' (3.6%). Respondents' religious affiliations reflect Malaysia's religious diversity: 779 (59.4%) of respondents were Muslims, 269 (20.5%) Buddhists, 126 (9.6%) Hindus, 115 (8.8%) Christians and 22 (1.7%) were other religions. Majority of the respondents have tertiary (688 persons, 52.5%) and secondary (584 persons, 44.5 %) education and 37.5% respondents (491 persons) earn RM1000 and below. Female respondents outnumbered male respondents: there were 720 females (54.9%) compared to 591 males. Six hundred and forty five respondents (49.2%) were in the category of '25 years and below' age group, with 373 (28.5%) in the '26-35 years'age bracket and the remaining 293 (22.3%) in the '36 years and above' age category.

The results showed 581 (44.3%) answered "yes" and 730 (55.7%) answered "no" to the question "Are you willing to donate your organ upon death?" Out of the 581 who said "yes", only 25 (4.3%) are registered organ donors. The following are the reasons of the 556 respondents (581-25) who still do not register as organ donors despite saying "yes" to donation (Table 1). The results clearly indicated that the reason "I do not know the procedure on how to register" recorded the highest score (285), followed by "No motivation whatsoever" (149). These observations suggested the importance of a concerted effort to inform the public not only on the importance of organ donation but also on the procedure of becoming a registered donor.

The results from the group who said "no" to be a donor (730) are presented in Table 2 and 3. Table 2 shows the reason why they said "no."

Under the structural considerations, two factors registered the highest score: the lack of accessibility to information and the lack of trust on the beneficial utilisation of their organ upon donation. Both scenarios reemphasize the above findings on the importance of channelling sufficient information to attract potential donors. The results clearly indicate that structural considerations (the lack of accessibility to information and the lack of trust on the beneficial utilisation of their organ upon donation) as opposed to religio-cultural considerations (against their religion and want their body to remain intact after death) scored the highest – 254 and 254 respectively.

Table 1. *Reasons not to be a registered organ donor by ethnic group*

Factor	Score				
	Malay	Chinese	Indian	Others	**Total**
I do not know the procedures on how to register	157	72	46	10	285
Still waiting for my family's consent	46	51	16	2	115
Due to time constraint	53	32	18	2	105
No motivation whatsoever	101	31	11	6	149
Others	60	45	17	12	134

Note: The respondents were allowed to choose more than one options, therefore the score presented is the total number of response for each factor.

Table 2. *Factors influencing respondents' decision not to be an organ donor by religious affiliations*

	Muslim	Christian	Buddhist	Hindu	Others	Total
Religio-cultural						
It is against my religion	68	5	24	5	-	102
I want my body to remain intact after death	113	9	24	5	2	153
Structural						
I do not have access to the information	187	13	40	11	3	254
I am not convinced that my organ/body part will be used beneficially	186	14	35	18	1	254
Others	122	15	42	11	6	196

Table 3. *Non-willing donors' attitude toward incentive and information by level of education*

Level of education	Factor	Yes	No	Undecided
Pre-tertiary	Tax rebate	52	201	93
	Adequate information is provided to respondents and their family.	74	211	99
Post-secondary	Tax rebate	70	159	117
	Adequate information is provided to respondents and their family.	90	170	124
	Total	286	741	433

Contrary to popular belief, religio-cultural is not the main impediment for people to become a donor compared to responds associated to structural considerations.

The results shown in Table 3 indicated that if the respondents, who were initially unwilling to donate their organs, were provided with adequate and complete information about the donation procedure, they will be more likely to be willing to donate, regardless of the level of education. In fact, the result shows that people are less likely to react to policies driven by monetary incentive, such as tax rebate compared to the desire for information.

When both level of education are combined, the results indicate that only 122 respondents were willing to change their mind to become a donor if tax rebate were offered but a greater number (164) would be willing to change their mind if complete information were given. It can be deduced from the results that monetary incentive may not be a sufficient inducement to one's decision to donate.

Discussion and Conclusion

The results bear some interesting conclusions. First, there is indeed a huge population of potential donor in Malaysia. Second, many citizens do not register for organ donation because they do not know the registration procedure. Third, the lacking of information and trust were the main reasons why they do not want to donate and, finally, people in Malaysia are more likely to change their view from not donating to donating their organs if adequate information were provided to them, rather than incentive.

The above findings may suggest that the lack of effective public education on organ donation may be the main cause of low organ donation rate and organ shortage in Malaysia; and elevating the investment in public education on organ donation may probably address the said problem.

Currently, conventional methods and strategies are commonly employed to educate the Malaysian public on organ donation. For instance, using living donors as role

models in campaigns, organising talk shows or organising sports event to create organ awareness. Meritorious as these efforts may be, these events have not turned in the result. As such, public education efforts hosted by the authorities and relevant stakeholders should not be limited only to organizing events that are macro in nature, but also activities that touches individuals at the personal level. One among the effective ways is to target the family, as studies have indicated that targeting individual families can better increase the chance of individuals to pledge for organ donation (11, 12, 13). The authorities may also target families of chronic patients, such as family members of dialysis patients. Employing such a niche approach could well influence one's decision to donate as family members of a chronic patients may be able to better relate to problems of organ failure. In fact, our preliminary research on this group has confirmed such claim; that is, the survey found that 80% respondents (family members of dialysis patients) were willing to be deceased donor while 35% of respondents had signed the organ donation pledge at the time the survey was conducted (14). Besides families of chronic patients, education on organ donation could also be meted out to care providers of chronic patients; medical staff and social workers who were closely associated with the patient.

Apart from the above, organising campaigns that appeal to the cultural, social and religious preferences of target communities could also increase organ donation pledges.

Effective public education could well complement possible policy decision to implement the "opt-out" rather than "opt-in" strategy that has proven to be successful in many advanced countries. Public education could also address issues relating to cultural-religio factors to allay fears on organ donation. Also more importantly, public education could well address Malaysia's palpable number of deceased organ donations which could prove to be the best option at addressing organ shortage.

The potential for organ donation in Malaysian population is largely unexplored. The current study indicates that insufficient information on organ donation and lack of trust towards the medical system are the main causes to low organ donation rate in the country. Re-evaluation and improvement of the current national programme and policies on organ donation to facilitate effective public education are thus crucial to enhance public awareness and acceptance towards organ donation, so as to address the problem of organ shortage in Malaysia.

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ANTI-RETROVIRAL THERAPY OF HIV INFECTED PATIENTS

Lubis R[1], Bulgiba AM[2]

1 Department of Epidemiology, Faculty of Public Health, University of Sumatera Utara, Medan, Indonesia
2 Julius Centre University of Malaya, Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Correspondence:
Rahayu Lubis
Department of Epidemiology
Faculty of Public Health (FKM)
University of Sumatera Utara (USU)
Medan, Indonesia
Email: rahayu@yahoo.com

ABSTRACT

Initiation of Highly Active Anti-Retroviral Therapy (HAART) depends on clinical or immunological criteria. Clinical criteria include the presence of opportunistic infections, categorized by the WHO as stage 3 and 4. Immunological criteria are based on CD4 cell count. The WHO guidelines have changed frequently. All patients with CD4 cell count less than 200 cells/µl and symptomatic HIV or late disease or severe recurrent HIV illnesses or patients with AIDS or tumor at any CD4 count, should start therapy. WHO guidelines in 2013 recommended initiating HAART at CD4 counts less than 500 cells/µl. HAART is usually initiated when CD4 is less than 200 cell/µl because HIV infected patients present at a late stage. Research on factors responsible for this is sorely needed so that interventions can be targeted at this group.

Keywords*: HAART, AIDS, HIV, CD4, Antiretroviral*

Highly Active Anti-Retroviral Therapy (HAART) has been available world-wide since 1996. It is a combination of three drugs which include 2 Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTI) or Non-Nucleoside Analogue Reverse Transcriptase Inhibitor (NNRTI) and Protease Inhibitor (PI) (1). Anti-retroviral therapy can help people living with HIV to live longer, increase quality of life and reduce AIDS-related deaths. According to the 2011 world report, anti-retroviral therapy is enjoyed by 8 million people, which is 20 times the number in 2003. Since 1995, anti-retroviral therapy has added 14 million life-years in low and middle income countries, including 9 million in sub-Saharan Africa in 2012 (2). HAART is given to decrease the viral load in the blood. This helps to repair the damage caused by HIV. It is recommended that a basic clinical assessment be carried out before starting anti-retroviral therapy. The clinical progression in HIV patients receiving treatment is estimated by the different levels of viral load and CD4 count. Existing medical conditions such as tuberculosis, hepatitis, injecting drug user (IDU), major psychiatric illness, pregnancy and body weight are identified to determine the patient's readiness for treatment. The presence of opportunistic infections have to be considered prior to starting treatment. A person's CD4 cells count too is used to determine when to start the treatment. CD4 cell count is also a major clue in asymptomatic patients (3). Several other factors such as symptoms, possible adherence, potential toxicity and patients' concerns should be considered in this matter (4).

All patients with CD4 cell count less than 200 cells/µl and symptomatic HIV or late disease or severe recurrent HIV illnesses or patients with AIDS or tumor at any CD4 count, should start therapy (3). If CD4 measurement is unavailable, simple tools such as haemoglobin level and total lymphocyte count can be used as laboratory markers to initiate HAART in resource-poor settings (5).

The question about when to initiate treatment should take into account that anti-retroviral therapy is a lifelong treatment, with significant adherence issues, potential side effects and high cost. To measure the HAART adherence, self-reported adherence (SRA) is an accurate instrument compared to therapeutic drug monitoring (TDM) and can be reliably used in practice in resource-poor settings (6).

The information about the prognosis of HIV infection is very important to monitor the progress of the HIV/AIDS epidemic, to develop treatment guidelines, to gain a better understanding of the prior treatment of HIV infection and to plan health services in the HAART period. These data are also important for the comparisons of treatment outcomes in resource poor settings once HAART becomes more widely available in less developed countries (7). Access to HIV care and anti-retroviral therapy still remain a challenge

to control the HIV epidemic in developing countries due to the financial burdens for people living with HIV in accessing and receiving HIV care (8).

Classes of drugs

There are different classes of antiretroviral drugs classified based on different phases of the retroviral life cycle and inhibitors. Anti HIV medications are grouped into six classes and each class targets a different step in the HIV life cycle (9,10).

Class 1: Nucleoside and nucleotide reverse transcriptase inhibitors (NRTI) inhibit the transcription back in a way incorporated into newly synthesized viral DNA and prevent further elongation.

Class 2: Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit the reverse transcriptase directly by binding to the enzyme and disrupting its function.

Class 3: Protease inhibitors (PI) targets viral assembly by inhibiting the activity of protease, an enzyme used by HIV to bypass nascent proteins for final assembly of new virions.

Class 4: Integrase inhibitors: inhibit the integration of the enzyme, it is responsible for integration of viral DNA into the DNA of infected cells.

Class 5: Entry inhibitors (or fusion inhibitors): disrupt binding, fusion and entry of HIV-1 into host cells by blocking one of several targets.

Class 6: Maturation inhibitors: inhibit the final step in the processing of *gag* in which the virus capsid poly-protein cleaved, thereby blocking the conversion of the poly-protein to the mature capsid protein (p24), because the virus particle has a core defect, which virions released consist mainly of non-infectious particles (9).

Using a combination of medications from different classes may increase the treatment's effectiveness while decreasing the risk of drug resistance. The approved medication to treat HIV infection fact sheet lists the food and drug administration (FDA) approved anti HIV medication by class, brand names, generic and date. Some are available as a combination pill of two or more different anti HIV medications from one or more classes (9).

Initiation of anti-retroviral therapy

The most important issue in HIV treatment is the determination of the optimal time to initiate anti-retrovirals. Anti-retroviral treatment can be initiated after an appropriate time of commencement and the combination of drugs to be used are decided. It will be influenced by many factors such as prior anti-retroviral history, stage of disease, concomitant therapies and illnesses, ability to tolerate and comply/adhere to certain combinations of drugs, adverse effects of the anti-retroviral agents, affordability and the cost of the regimen.

It is very important to ensure that the patient is able to adhere to the anti-retroviral regimen that he/she is started on. The importance of reducing the development of resistant viral strains and good drug adherence in maintaining viral suppression was highlighted in previous studies. Thus antiretroviral therapy should only begin when the patient is committed to long term treatment (9). The guideline recommends that in developing countries, HIV infected adults and adolescents should start anti-retroviral therapy when one of the following conditions is present and HIV infection has been confirmed.

Generally, the decision to start anti-retroviral therapy depends on the clinical or immunological criteria. Clinical criteria are based on the presence of one or more severe opportunistic infections, categorized by the WHO as stage 3 and 4. All developed and developing country guidelines recommend starting anti-retroviral therapy if a patient presented with stage 3 or 4 though decisions based on such clinical criteria alone are generally only used in resource limited settings where laboratory capacity is limited (1). Commonly, the decision to start anti-retroviral therapy is based on immunological criteria, as defined by the level of CD4 count. According to current WHO guidelines 2013, all HIV-infected patients with a CD4 less than 500 cells/µl must be started on HAART (11).

The majority of the current HAART regimens consist of 2 NRTI + a NNRTI/PI. The first line drug which is used in initial regimens must have low side effects and high efficacy. Treatment guidelines for adult HIV-1 infected patients in the developed countries have been provided by the International AIDS Society (IAS) USA since 1996. The IAS-USA anti-retroviral guidelines therapy was developed by a panel of volunteer experts.

Resistance tests are recommended prior to initiation of treatment. This is important for urgent treatment needs and to handle high rates of baseline resistance in certain countries. Later the chosen treatment regimen can be started and adjusted further based on resistance test. In Britain, there are 11.8 per cent of moderate to high level of resistance at baseline to a combination of Efavirenz + Zidovudine + Lamivudine and 6.4 per cent for medium to high level resistance to Stavudine + Lamivudine + Nevirapine (12).

In early 2010, the European AIDS Clinical Society (EACS) guidelines stated that in HIV-infected patients with CD4 count 350 to 500 cells/µl treatment should be considered if there is a viral load of more than 100,000 copies/ml, if CD4 declines to more than 50-100 cells/µl per year, age more than 50 years, high cardiovascular risk and malignancy. For patients with CD4 having more than 500 cells/µl, anti-retroviral therapy should be deferred. Treatment can be offered in the presence of co-morbid condition or in patients seeking and are ready for anti-retroviral therapy (13).

In July 2010, the panel of the International AIDS Society (IAS) in the US recommended anti-retrovirals for all HIV-infected patients with CD4 counts up to 500 cells per microlitre and in those with 500 cells per microlitre but were losing CD4 at a rate of more than 100 cells per microlitre per year, had viral loads more than 100,000 and whose ages were more than 60 years (14).

The guidelines of when to begin anti-retroviral therapy has been changed frequently (1). A comparison of anti-retroviral therapy initiation based on WHO guidelines in 2013, 2010 and 2006 is summarized in Table 1.

Switch to second line anti-retroviral therapy

Second line HAART regimes are indicated for patients who are forced to discontinue their initial treatment regime as a consequence of treatment failure or severe toxicity. Treatment failure is defined by clinical failure, immunological failure or virological failure (11). WHO definition of clinical failure for adult and adolescents is new or recurrent clinical event indicating severe immunodeficiency (WHO clinical stage 4 condition) after 6 months of effective treatment. Immunological failure is defined as CD4 count falling to the baseline (or below) or persistent CD4 levels below 100 cell/µl. Virological failure is defined by persistently detectable viral load exceeding 1000 copies/ml (that is two consecutive viral load measurements within a three months interval, with adherence support between measurements) after at least 6 months of using ART (11).

The recommendation of WHO (2013) when to switch to second line ART is based on:

- Where available, use viral load to confirm treatment failure;
- Where routinely available, use viral load every 6 months to detect viral replication;
- A persistent viral load more than 1000 copies/ml confirms treatment failure;
- When viral load is not available, use immunological criteria to confirm clinical failure;

Anti-retroviral therapy switching criteria (11):

- Clinical failure: new or recurrent stage 4 condition (condition must be differentiated from an immune reconstitution inflammatory syndrome/IRIS; certain WHO clinical stage 3 conditions (PTB, severe bacterial infection) may be an indication of treatment failure;
- Immunological failure:
  - Fall or immunological to baseline or below;
  - 50 per cent fall from on treatment peak value;
  - Persistent CD4 level below 100 cells/µl;
- Virological failure: viral load of more than 1000 copies/ml, the optimal viral load threshold for defining viral load failure has not been determined. Values of viral load which are more than 1000 copies/ml are associated with clinical progression and a decline in the CD4 cell count. The targeted viral load strategy for failure and switching anti-retroviral therapy is displayed in Figure 1.

Table 1. *A comparison of anti-retroviral therapy initiation based on WHO guidelines in 2013, 2010 and 2006*

Target population	2013 ART guidelines	2010 ART guidelines	2006 ART guidelines
HIV + asymptomatic	≤ 500 cell/µl	≤ 350 cell/ µl	≤ 200 cells/ µl
HIV + symptomatic	WHO clinical stage 1 and 2 if CD4 ≤ 500 cell/µl (CD4 ≤ 350 cells/ µl as priority)	WHO clinical stage 2 if CD4 ≤ 350 cells/µl WHO clinical stage 3 or 4 irrespective of CD4 cell	WHO clinical stage 2 if CD4 ≤200 cells/µl WHO clinical stage 3 if CD4 not available WHO clinical stage 4 if CD4 irrespective of CD4 cell Consider treatment for WHO clinical stage 3 and CD4 cell between 200 and 350 cells/µl
HIV + pregnant	All pregnant and breastfeeding women should initiate triple ART and maintained at least for the duration of MTCT risk. Women meeting treatment eligibility criteria should continue lifelong ART	CD4 < 350 cell/ µl irrespective of CD4 cell orWHO clinical stage 3 or 4 irrespective of CD4 cell	WHO clinical stage 1 or 2 if CD4 <200 cells/µl WHO clinical stage 3 or 4 if CD4 < 350 cells/µl WHO clinical stage 4 irrespective of CD4 cell
HIV/TB co-infection	Presence of active TB disease, irrespective of CD4 cell	Presence of active TB disease, irrespective of CD4 cell	Presence of active TB disease and CD4 ≤350 cells/µl ART Initiation can be delayed if CD4 ≥200 cells/µl
HIV/HBV co-infection	Individuals co-infected with HBV with evidence of severe chronic liver disease	Individuals who require treatment for their HBV infection, irrespective of CD4 cell count	No specific recommendation

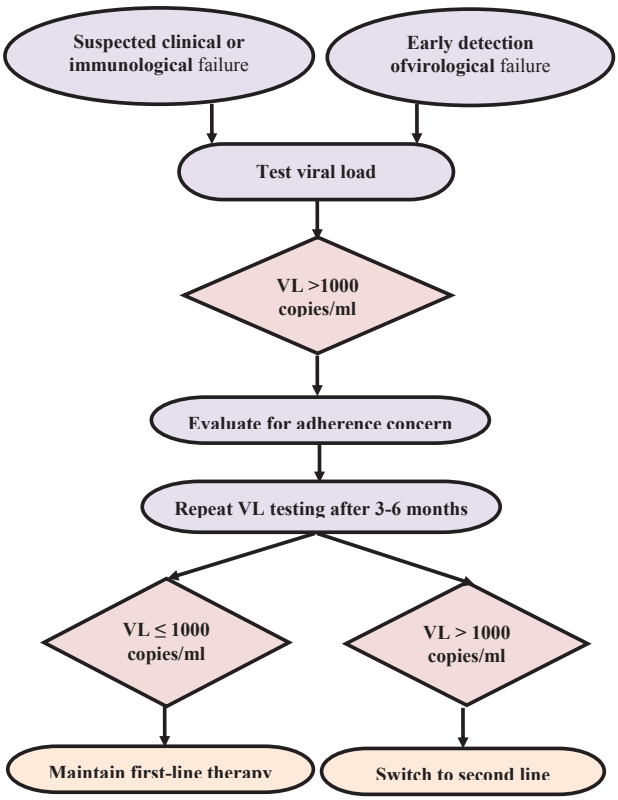

Figure 1. *Targeted viral load strategy for failure switching*

Second-line drug regimens

The 2010 WHO ART guidelines recommended that second line regimens included a boosted PI plus two NRTIs (determined by the drug used in first line therapy). Those guidelines placed a high value on using simpler second line regimes, ideally heat-stable formulations and fixed-dose combinations (one-daily formulation when possible). Since first line ART should preferably be based on an NNRTI, PI based regimens are recommended for second line therapy. Of the PI option, Atazanavir/Ritonavir (ATV/r) and Lopinavir/Ritonavir (LPV/r) are preferred. Darunavir/ Ritonavir (DRV/r) is an alternative but is currently not available as a fixed-dose combination, although one is in development. The other PIs such as Fosamprenavir/ Ritonavir (FPV/r), Indinavir/Ritonavir (IDV/r), Saquinavir/ Ritonavir (SQV/r) are not available as heat-stable fixed-dose combinations and/or associated with high pill burden and higher frequency of side effects. Second line ART regimes for adults and adolescents; if Stavudine (d4T) or Zidovudine (AZT) was used in first line, preferred second line regimes are Tenofovirdisoproxilfumarate (TDF) + Lamivudine (3TC) or Emtricitabine (FTC) + ATV/r or LPV/r. If TDF was used in first line ART, preferred second line regimen are AZT + 3TC + ATV/r or LPV/r (11).

Anti-retroviral therapy in Malaysia

In Malaysia, anti-retroviral treatment is available at all general hospitals and some district hospitals where there are specialist physicians managing medical clinics. Anti-retroviral treatment is also provided in some local university hospitals. All HIV clinics are run by physicians, who have had some training in HIV medicine.

The standard HAART Regimens (1;4) comprise one of three possible regimens:

- NRTI + NRTI + PI
- NRTI + NRTI + NNRTI
- NRTI + NRTI + PI + PI

Anti-retroviral therapy was introduced in stages in Malaysia. In 1989, AZT was available, in the early 1990's ddI and ddC were also available and in mid-1990's d4T and 3TC became available. In Malaysia, HAART was available from February 1997 with the entry of IDV but the cost of these drugs was high. In the late 1990's, a standard regimen of AZT + 3TC + IDV, would cost close to RM 2000 per month. In 2003, the MOH took the initiative to access cheaper generic anti-retroviral drugs from India. This led to the introduction of generic d4T, RTV and NVP into Malaysia. Generic drugs such as AZT, ddI and the fixed drug combination of AZT + 3TC, followed subsequently in early 2004. This led to the second big jump in anti-retroviral therapy uptake locally (4).

One of the three targets of Malaysia's Millennium Development Goals (MDG) is to achieve treatment for all HIV/AIDS patients by 2010. The Ministry of Health Malaysia reported that 9,962 people living with HIV had received anti-retroviral therapy by the end of 2009 (15). The Government of Malaysia provides first line anti-retroviral access for all HIV-infected patients without charging money at all government hospitals and clinics. From 2006, the Government of Malaysia provided anti-retroviral therapy to all HIV patients in prisons and drug rehabilitation centres. The second line anti-retroviral therapy is partly subsidized by the government (15). Malaysia will continue providing affordable access to clinical care for HIV patients through the public health system (4).

Anti-retroviral therapy programs in developing countries follow a public-health approach rather than an individualized approach. Guidelines for developed countries cover individual patient management delivered by specialist doctors prescribing from the full range of anti-retroviral drugs, supported by routine high-technology laboratory monitoring. This approach is not suitable in resource-limited settings where doctors are scarce, laboratory infrastructure is not adequate and the procurement and supply-chain management is frail. This difficulty in translating guidelines from developed to developing nations caused concerns over whether anti-retroviral therapy scale-up in poor countries is feasible, affordable and cost-effective (16).

Most HIV infected patients present at a late stage in developing countries and HAART is usually initiated when CD4 was less than 200 cell/µl, although WHO guidelines in 2013 recommend HAART initiation when CD4 is less than 500 cell/µL. Research on factors responsible for this is sorely needed so that interventions can be targeted at this group.

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LIST OF REVIEWERS FOR VOLUME 17, ISSUE 1, 2014

Associate Professor Dr. Aziz Nather

Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine,
National University of Singapore, Singapore

Associate Professor Dr. Suzina Sheikh Ab Hamid

Department of Otorhinolaryngology -Head & Neck surgery (ORL-HNS),
School of Medical Sciences, Healthy Campus,
Universiti Sains Malaysia, Kelantan

Ybhg. Prof. Dato' Dr. Tunku Sara Binti Tunku Ahmad Yahaya

Department of Orthopaedic Surgery, Faculty of Medicine,
University of Malaya, Kuala Lumpur

Dr. Jutarat Praparattanapan

Department of Medicine, Faculty of Medicine,
Chiang Mai University, Thailand

Associate Professor Dr. Keivan Zandi

Department of Medical Microbiology, Faculty of Medicine,
University of Malaya, Kuala Lumpur