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Correspondence

All manuscripts, general correspondence and enquiries should be addressed to: Journal of Health and Translational Medicine (JUMMEC), The Dean's Office, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, MALAYSIA.

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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 Articles, Original Articles, Short Communications, Clinico-pathological conference abstracts, Case Reports, Letters to the Editor and Book Reviews.

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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.

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Transfer of fresh obtained blood into EDTA tube. Image courtesy of Sangeetha Vasudevaraj Naveen

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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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Each manuscript component must begin on a new page in the following sequence: (1) title page; (2) abstract and keywords;

(3) text; (4) acknowledgements; (5) references; (6) figure legends;

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The title page should contain a concise title of the article. Names of authors who have contributed to the writing of the manuscript should be written in style of initials followed by surname or preferred name, eg. Saleena VEO, Anita S or Brown J. Add at the bottom of the phrase "Address for correspondence;" followed by full name and address with postal code and email address.

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It should consist of an Introduction, Methods, Results, Discussion and Conclusion/Recommendation. Systeme Internationale (SI) Units should be used. Use only standard abbreviations. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement.

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Number the references in the order of mention in text. References in the text should be indicated by a figure within parenthesis e.g. (1, 2,). Limit references to 30, if possible. Identify references in text, tables and legends.

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Forewords from the Editor-in-chief

Dear Readers of JUMMEC,

Welcome to the Journal of Health and Translational Research's *(JUMMEC)* 2nd issue published in the year 2011. In this issue, we are happy to present to you several interesting articles for your reading pleasure. In this issue two articles with regards to medical students are described each relating to a particular aspect of cohorts of that age. These article covers either academic achievement process or health risk behavior assessment, which is described in great detail. It is of interest to note that in the latter article, medical students who are aware of health risk issues as the result of promiscuous behavior still actively engage in unhealthy practices. This study does suggests that knowledge alone is insufficient to prevent or reduce unhealthy conducts amongst people of that age. The other two lab based results article featured are reference articles which describes the process of tyrosine assay optimization whilst the other article is on the optimization of platelet rich plasma (PRP). PRP today is a hot topic and is mentioned widely by the public namely in the areas of tissue regeneration. Hence, the publication of this article is timely since it will provide readers important information which will help them to understand the process by which PRP is produced.

It is our hope that the articles featured in this issue will inspire the readers to pursue better research in an attempt to strive for excellence in areas of health and translational medicine.

Happy Reading!

With best wishes,

Tunku Kamarul Zaman,
Editor-in-chief,
The Journal of Health and Translational Medicine.

PERFORMANCE OF THE FINAL YEAR MEDICAL STUDENTS IN UNIVERSITI MALAYSIA SABAH IN THE END SURGICAL SENIOR POSTING (SSP) EXAMINATION

Almothafar B[1], Chee FT[2]

1 Department of Surgery, School of Medicine, University Malaysia Sabah, Sabah
2 School of Sustainable Agriculture, University Malaysia Sabah, Sabah

Correspondence:

1 Basim A. M. H Almothafar
Department of Surgery, School of Medicine, University Malaysia Sabah,
Locked Bag 2073, 88999, Kota Kinabalu, Sabah, Malaysia
E-mail: basim_amh@yahoo.com
2 Chee Fong Tyng
School of Sustainable Agriculture, University Malaysia Sabah,
Locked Bag 2073, 88999, Kota Kinabalu, Sabah, Malaysia
E-mail: ftchee@ums.edu.my

ABSTRACT

The present study reports the performance of final year medical students from the Universiti Malaysia Sabah (UMS) in the end of the senior surgical posting examination (SSP) with the aim to demonstrate the medical students graduating from this newly established university are of good standing and of improving quality. A study on the outcome of the method of teaching conducted on this study was performed by measuring the students' performance continuously and at the end of their posting. The present data analyses demonstrate that there have been improvements in the medical students' performance between the last two batches of students graduating from UMS. However, the students appear to be weaker in their MCQs, demonstrating a decline in theoretical knowledge. The analyses also demonstrate that there is a poor positive correlation between theoretical knowledge, clinical skills and/or continuous assessments, demonstrating the importance of emphasis in these 3 areas amongst medical students. Further studies may be required to determine the reason for this poor correlation since these may lead to better understanding on how to improve the overall performance of future medical student. CONCLUSION: The present study demonstrates that UMS medical student appears to continue to improve in their SSP performance although there are concerns about the decline in theoretical knowledge.

Keywords: *Clinical assessment, performance-assessment, senior surgical posting, MCQ, MEQ, Essay, OSCE, Short case, long case.*

Introduction

Performance is said to be the product of complex relationships between skills and knowledge, mediated by perceptions of anxiety, self-confidence and preparedness *(1)*. It has been demonstrated that the mode of assessment influences the learning style of students (assessment-driven learning), and that it has been shown that medical students are susceptible to this influence *(2)*. It is thus necessary to bring the theories of learning and assessment together. During their period of enrolment, students are expected to develop: (1) a higher level of conceptualization at the end of the learning process than at the entry level, (2) an ability to relate one part of what they had learnt to another, and (3) an ability to retrieve knowledge in appropriate situations *(3).*

In many medical schools, senior surgical posting is an intensive eight-week attachment, which includes an overall surgical revision period. It provides an opportunity for the students to develop clinical skills, knowledge and attitudes. At the end of the posting, the students will be evaluated by three forms of assessment, their knowledge by MCQs, MEQs, and Essay and their clinical skills by OSCE, short case and long case examination in addition to students' assessment (log book viva and two handwritten case

reports). The passing mark is 65 of 100. Passing the clinical section is mandatory. The primary objective of this rotation is to ensure that the final year students will develop sufficient knowledge that will enable them to deal with most of the surgical emergency and problems encountered during their clinical practice. This eight weeks posting will provide an opportunity for the students to develop their clinical skills, knowledge and attitudes through practical sessions on the wards, outpatient clinics, in the emergency room and in the operating theatre.

The course also provides a framework of structured didactic teaching on some of the common surgical problems such as lectures, ward rounds (pre-operative and post-operative), clinical skill laboratory, seminars and tutorials in the relevant aspects of clinical laboratory sciences. During the didactic teaching, students are attached to the general surgical department for five weeks, then to each of the emergency and neurosurgical and paediatrics departments for one week together with that, the students will spend time in the theatre and surgical clinic. At the end of this period, the students will sit for the end posting examination for which an overall assessment of their performance will be measured in a single exam sitting.

In view of the importance of this posting and that of the influence and role the teaching program that is being used towards the development of the medical education, an analyses of the results of final year medical student following 8 weeks in posting was conducted. Several research questions were being posed in the present study; Did the students meet the educational objectives of senior surgical posting program especially the skills and knowledge and in which domain are their weakness.

Materials and methods

Retrospective data collected and analysed from the end surgical senior posting examination (SSP) for the final year medical students from the school of medicine UMS academic year 2008-2009 (36 students) and academic year 2009-2010 (69 students) were collected from our records. In their training, students were divided into six blocks, each spent eight weeks in the senior surgical posting (SSP) and assessed at the end posting examination.

At the end of the posting, the students were evaluated in a three-day exam continuously. The first day was for the assessment of the knowledge through three mode of assessments, i.e. MCQs, MEQs, and Essay. There were 30 questions for the MCQs.The score for each correct answer is one mark, incorrect answer is deducted a points and unanswered question receives no points. The second and the third days were for clinical assessments by various examiners. The assessment had three methods (OSCE, Short case, Long case). In OSCE examination, the students were evaluated at ten different stations. Each station had clinical signs photos or investigation data or medical instrument. The students were tested in a variety of skills including interpretation of clinical signs, formulation of a diagnosis from clinical and laboratory information. Two examiners assessed the students in a long case format. Both assessed the student's presentation of the history, clinical examination and the discussion on the management of the case. In short case assessment format, the students were requested to perform clinical examination for specific medical conditions and evaluated through her/his ability to elicit and interpret the physical signs. In logbook viva, two examiners evaluated each student for fifteen minutes by passing through all the activity of the student during his/her course. Two handwritten case reports were evaluated by the examiners. The report was 5 marks each. The evaluation was based on the methodology of the reports (history, physical examination and discussion on the management). On the overall, a minimal passing mark of 65 of 100 would be necessary to pass the exam, and that a passing in the clinical section is mandatory.

In order to simplify the analysis, both log book viva and the two handwritten case reports data were placed under one category called continuous assessment, whilst the remainder were placed as single end assessments. Analyses of the single end assessments were performed at 2 levels. The first level include the measurement of the means for the three major components (theory, clinical and continuous assessment). In the second level, the means for the theory components (MCQs, MEQs and Essay) and clinical components (OSCE, Short case and Long case) were

Table 1: Structure of examination for SSP.

No.	Question types	No. of questions	Time (Minutes)		Mark (%)
			Per Question	Total per Exam	
1	Log book	-	-	-	30
2	Two Handwritten Case Reports	-	-	-	10
3	Multiple Choice Questions (MCQs)	30	2	60	10
4	Modified Essay Questions (MEQs)	12	5	60	10
5	Essay	2	30	60	10
6	OSCE	10 Stations	5	60	10
7	Long case Examination	1 Station	-	90 (clerking 60 minutes)	10
8	Short case Examination	2 Stations (at least)	10	20	10

measured and compared separately. Analyses included simple correlation and regression analyses to examine the relationship between various components of the examination. The analyses were conducted using statistical software package SPSS Version 17.

Results

During the two academic years (2008-2009 and 2009-2010), 105 students were examined at the end of the SSP, and all of them passed the examination. Table 2 shows the mean scores for each examination component for each of the two academic years and for the two years combined. The table summarizes the mean overall total score for both years and that it demonstrate significant differences between these scores ($p<0.05$). The mean score for the MCQs component for academic year 2008-2009 is higher than academic year 2009-2010, but for MEQs the mean score for academic year 2008-2009 was lower than academic year 2009-2010.

The performance of 2009-2010 students in the short cases and OSCE was higher than that of the 2008-2009 students. However, Essay questions and long cases examination showed no significant difference between these batch of students ($p>0.05$). A histogram (Figure 1) illustrates the performance of the students between the two batch of students. There is a clear indication that the scorings had improved between the two graduating batches (with most students grades increased to grades A and A-). However, this is only observed when the theory component was excluded from the analyses. Their performance was remarkably reduced when the continuous assessment component was excluded from the analyses (most of them were between grades B+, B and B-).

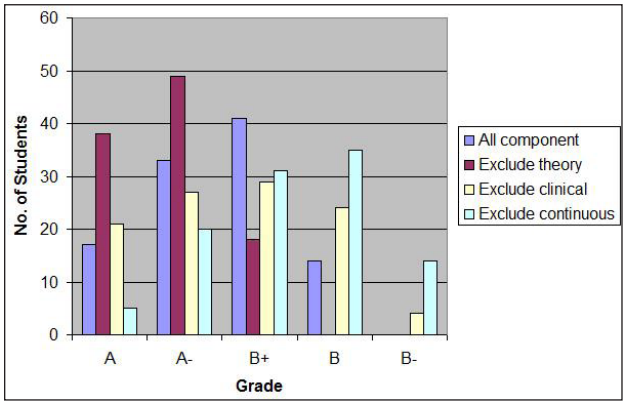

Figure 1: Histogram showing the distribution of the scores for the 105 students as a percentage when all the components are taken together, when the theory component is excluded, when the clinical component is excluded, and when the continuous assessment is excluded.

The score percentages are divided based on UMS grading system: 60 – 64% (B-), 65 –69% (B), 70 –74% (B+), 75 – 79% (A-), 80 –100% (A).

Table 3 show that the performance of the students in the theory part of the examination (mean score=65.80) were weak in comparison to the other two parts, the clinical (mean score=76.20) and continuous assessment (mean score = 80.60). In table 4, the component of the theory assessment, performance of the students in answering MEQ (mean score = 7.52) and Essay (mean score =7.51) questions demonstrated improvements. However students were weak in their MCQs (mean score =4.70). Table 5 show that the components of the clinical examination performance of the students were equally well in OSCE (Mean score = 7.82)

Table 2: Mean (SD) score for each examination component during each of the two academic years and for the group as a whole.

Academic Year	MEQs (M=10)	MCQs (M=10)	Essay (M=10)	OSCE (M=10)	Short Cases (M=10)	Long Cases (M=10)	Continuous (M=40)	Overall total score (100)
2008-2009	6.77a	5.39a	7.38	7.65a	7.12a	7.74	32.93a	74.97
(S=36)	(0.74)	(0.78)	(1.03)	(0.61)	(0.87)	(0.94)	(2.14)	(4.55)
2009-2010	7.92b	4.34b	7.59	7.91b	7.47b	7.64	31.88b	74.76
(S=69)	(0.96)	(1.08)	(1.04)	(0.42)	(0.68)	(0.70)	(2.01)	(4.55)
all group	7.52	4.70	7.51	7.82	7.35	7.68	32.24	74.83
(S=105)	(1.05)	(1.11)	(1.04)	(0.50)	(0.76)	(0.79)	(2.11)	(4.53)

M = Maximum score for each component of the examination
S = Number of students
Different alphabet after number indicates significant difference at $p<0.05$, tested between years

Table 3: Comparison between Theory, Clinical and Continuous Assessments

	Mean score (per 100%)*	Std. Deviation	Std. Error	Minimum	Maximum
Theory	65.8[a]	7.83914	0.76502	51.67	81.67
Clinical	76.2[b]	4.87976	0.47622	58.00	89.00
Continuous	80.6[c]	5.26278	0.51359	68.50	93.75
Total	74.1857	8.71782	0.49119	51.67	93.75

* Different alphabet after number indicates significant difference at $p<0.05$

and long case (Mean score =7.68). However, the students' performance in the short case section (Mean score =7.35) were lower than the other two components.

Table 4: Comparison between the theory components.

Theory Assessment component	Mean (/10%)*	Std. Deviation	Std. Error	Minimum	Maximum
MEQs	7.5229b	1.04562	0.10204	5.10	9.70
MCQs	4.7029a	1.10684	0.10802	2.10	6.90
Essay	7.5143b	1.03677	0.10118	5.10	9.70
Total	6.5800	1.70040	0.09581	2.10	9.70

* Different alphabet after number indicates significant difference at p<0.05

Table 5: Comparison between clinical components.

Clinical Assessment components	Mean (/10%)*	Std. Deviation	Std. Error	Minimum	Maximum
OSCE	7.8229b	0.50483	0.04927	6.50	9.00
Short case	7.3467a	0.76297	0.07446	3.90	9.50
Long case	7.6762b	0.78551	0.07666	4.70	9.40
Total	7.6152	0.72205	0.04068	3.90	9.50

* Different alphabet after number indicates significant difference at p<0.05

By using simple correlation and regression analysis to study the relationship between the three major components (Theory, Clinical and Continuous assessment), it was found that there was weak positive association between theory and clinical (r=0.39; p<0.05; r^2=0.15), between theory and continuous assessment (r=0.44; p<0.05; r^2=0.19), and between clinical and continuous assessment (r=0.23; p<0.05; r^2=0.06) (Figure 2 - 4). Therefore a weak association between the three major components are of concern which needs to be further investigated. We speculate that this may be caused by other factors other than knowledge and skills; most likely influenced by external factors such as psychological factor.

Discussion

The three major components

The results indicate that the performance of the students in continuous assessment was the higher than in clinical or theory assessments. However how is the process of continuous assessment reliable? Continuous assessment is considered to be a form of global rating (which describes any summative judgement of a student's performance completed by a supervision after a period of contact) *(4)*.It consist of two parts, one is the log book when the students record their activities in the surgical department, then assessed by one or two examiners through a viva voce. The supervisors whom are supposed to supervise the students are the consultant surgeons, specialists, medical officers and even house officers working in the surgical department. Thus theoretically a medical student may be supervised by multiple levels of supervisors, and of different levels of competencies. Despite being widely employed, studies on the use of global ratings that has been discussed and reviewed extensively showed that they are generally considered to be unreliable as an assessment method *(4).*

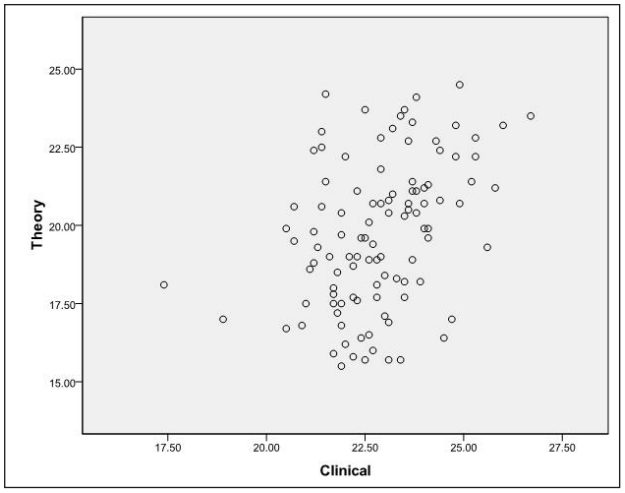

Figure 2: Correlation and regression between theory and clinical.

Together with that, the oral examination of this part of assessment is also criticized. The application of oral examinations in education has been reviewed, and it has been demonstrated to have low reliability as assessments of clinical competence. Also there is a consequence of low reliability between examiners (inter-rater reliability) where some examiners tend to mark generously (doves) and some have a tendency to award low marks *(4)*. In concern of the validity of oral ratings, studies indicate that the mark awarded to a candidate may reflect factors other than the candidate's clinical competence; namely anxiety, percentage of words contributed to the discussion by the candidate, the examiner's visual impressions of the candidate or the candidate's self-confidence *(4)*.

The second part of the continuous assessment is a two-case report where the students have to prepare during his/her attachment, then evaluated by one of the examiner. His/her evaluation will depend on that examiner, i.e. different examiners evaluating the case reports, will also affect the reliability of this assessment.

The passing mark in this posting is 65 of 100.In order to evaluate how continuous assessment has a role in the final result, continuous assessment marks are excluded from the total assessment and the other two components (theory and clinical) are revaluated, we found that 14 students of the total (14.7%) have failed to reach the passing marks (39 of 60).

Forty marks are given to continuous assessment. This assessment is a strong factor to support the final marks of the students e.g. a student scored 38.2 (out of 60) in both clinical and theory but scored 35.5 in continuous assessment, and this helped the student to gain 73.7 (B+). While a student scored 35.5 (of 60) in both clinical and theory but scored 32.3 in continuous assessment, the final mark is 67.8(B) that enable the student to pass the end posting examination.

Theory components

The other data are the three components of the theory assessment MCQ, MEQ and Essay questions. The students performance in MCQs was the lowest and the mean of all the students in MCQs = 4.7029 which was lower than 5 of 10 marks.

The MCQs examination was perceived as assessing knowledge-based or lower levels of cognitive processing and the assignment essay was perceived as assessing higher levels of intellectual skills and abilities such as analysis, application and comprehension. MCQs are reliable, easy to mark, and can be used to sample a large part of the curriculum, and to discriminate between candidate's levels of knowledge *(4,5)*. In other hand the considerable disadvantages of essays for assessment are easy to set, difficult to mark and have low reliability, such exams encourage strategic learning, question-spotting and lead to undesirable learning patterns. *(4)*

The MCQs reflect the real theory background of the students. The weak performance of the students in MCQs could result from:

1- poorly designed questions e.g. using complex stem which may require a degree of analysis (context-dependent multiple-choice questions) could be considered as difficult for the student's level.

2- negative marks given for incorrect answers. Is it fair for all types of questions to be given minus one mark for each incorrect answer? These negative marks should be designed according to the value of the questions itself e.g. questions that answered by fatal mistakes are different in it's evaluation from that questions that were not changed the management of the patients, some educational centres regard minus 0.25 – 0.5 marks as penalty for the incorrect answer *(5)*.

3- students study approach .Most MCQs tests are factual recall of information and this need the students to have a good theory background. Research on learning in higher education suggests that students have a preferred approach in their studies, usually referred to as either a deep approach (focusing on meaning and understanding) or a surface approach (focusing on recall and reproduction). Students were significantly more likely to employ surface learning approached (surface strategies and surface motives) when preparing for their MCQs examination, and deep learning approaches (deep strategies and deep motives) when preparing their assignment essay *(6).*

An approach consists of a strategy and a motive. For example, a student employing a deep approach might integrate the theoretical and practical components of a course (deep strategy) with the intention to understand and make sense of the material (deep motive). In contrast, a student employing a surface approach might list and drill several discrete pieces of information (Surface strategy) in order to reproduce them in the examinations and pass the course (surface motive) *(7)*. The employment of both surface and deep strategies could result in good test scores *(6).*

Figure 3 reflects this fact for the Students School of Medicine-UMS. It shows that most students preferred deep approach studies. Thus their performance in MEQs & Essay was higher than in the MCQs performance which request a good theory background (surface approach studies). i.e. They are not spending enough time on the theory part of the course.

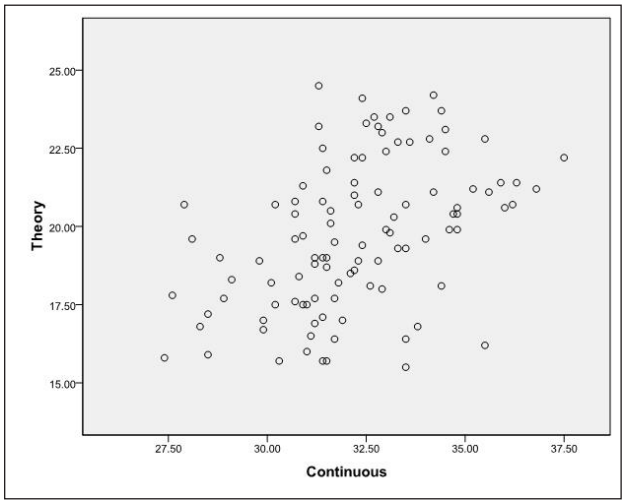

Figure 3: Correlation and regression between theory and continuous.

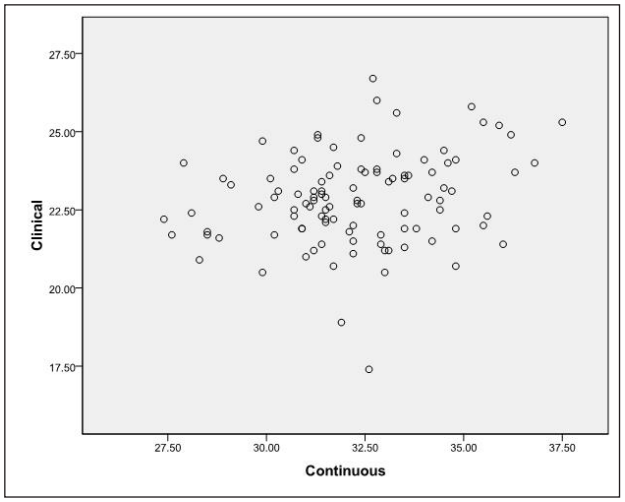

Figure 4: Correlation and regression between clinical and continuous.

Clinical components

The examinations for the clinical skills programme were competency-based. They were designed to assure minimal performance standards. The relationship between knowledge base and clinical skills also might reflect that clinical skills mastery builds upon a biomedical knowledge base *(1)*.

There is no big difference in the performance of the students in the three forms of clinical section of the assessment. However their performance in the short case were lower than the other two components. Individual performance shows that two students of total 105 failed to pass long case (2.1%), and eight of them failed to pass the short case (8.4%), while all of them pass the OSCE. In my opinion, the higher percentage of failure short case could result from:

1) weakness in the performance of physical examination and eliciting the physical signs.
2) psychological impact, during the short case in which the students have to show their skillsin front of the examiners in a short time (10 minutes). The impact is greater during the short case than during the long case which is practically a theory discussion on the patients history and management in reasonable time (one and half hour). While such impact is lower during OSCE when there is no examiner supervising the students directly.

The relation between psychological status of the students and their performance have been discussed in many papers. Brian Mavis (2001) reported that students performance in the clinical skills and biomedical science curriculum were related to perceived anxiety, which was related to self-efficacy. Preparedness was predicted in self-efficacy and itself predicted performance. Knowledge also had a strong direct link to performance *(1)*.

Jo-Ann (2006) indicated that the students with low levels of test anxiety achieve higher scores on MCQs examinations than those with high anxiety levels. Female students have been shown to have higher test anxiety levels than male students. [7] Is it helping the students by integrating stress-reducing programmes into medical school curriculum? Although there is evidence that students participation in stress reducing programmes does improve test scores and demonstrate increase in empathy and sensitivity towards patients. This may also help medical schools to better understand the learning process *(7)*.

Together with that the good performance in OSCE assessment was due to the OSCE form used in assessing UMS-students. It consists of 10 stations of clinical signs (photos) or clinical data (e.g. laboratory data, X-ray) without patients stations. So the psychological impact of OSCE form here is less than in short and long case examinations when the students have to face patients and examiners. In designing an assessment method in clinical examination, examiners need to put real situations in consideration as the students in real life, that is when they become doctors, will deal directly with the patients.

The objective structured clinical examination (OSCE) has been shown to be a valid and reliable assessment instrument for clinical competence in a comprehensive, consistent and structured manner *(8)*. However the absence of the patient station in the OSCE form that was used by the UMS medical students put this assessment into critical situation.

Conclusion

The present study demonstrates that UMS medical student faired better over the subsequent years with an increase observed in their SSP performance in all major components of the assessment process. However the worrying trend in terms of the declining MCQ scores needs to be heeded, which indicates that the theoretical knowledge of students is declining. Further inconsistencies of the loss of correlations between major assessment components of SSP also needs to be look into objectively so as to ensure that better performance of the students can be achieved.

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HEALTH RISK BEHAVIOUR AMONG UNDERGRADUATES IN A MALAYSIAN PUBLIC UNIVERSITY: A CROSS-SECTIONAL STUDY

Liew HC, Noor Illiati I, Raymond Yii SL, Siti Nadzrah Y, Moy FM

Faculty of Medicine, University of Malaya, Kuala Lumpur

Correspondence:

Liew Hoe Chin
95, Taman Makmur, Off Jln. Tun Dr. Ismail, 70200 Seremban, Negeri Sembilan.
E-mail: maximilienliew@hotmail.com

ABSTRACT

INTRODUCTION:
Health-risky behaviours among undergraduates are a course of concern both due to its tendency to be carried towards later adulthood and the emergence of non-communicable diseases at younger age group.

METHODS AND MATERIAL:
A cross-sectional study involving 1622 respondents comprising of students undertaking tertiary education was carried out using self-administered questionnaires. (adapted and translated into Bahasa Malaysia from the CDC Health Risk Behaviour Survey)

RESULTS:
It is found that 6.9% of the respondents have had sexual intercourse with the mean age of the first intercourse at 18.6 years. Of those who have had intercourse, 25.9% did not practice safe sex. The study showed that 7.2% of the undergraduates who drives have driven after taking alcohol and 19.3% of the respondents have travelled in a car driven by drunk driver. It is also found that 10.8% of the respondents tried smoking at a young age of 15 years. One point two percent (1.2%) of the studied population has also taken recreational drugs previously.

CONCLUSION:
Health risk behaviours among undergraduates in Malaysia are presently existing issues which poses serious concerns.

Keywords: *tertiary students, sexual intercourse, substance abuse, recreational drugs, smoking*

Introduction

Undergraduates undergo a transitional state from being under parental supervision to later full adulthood independence. As undergraduates are at this stage being exposed to new environments, ideas, freedom and companies of friends, there is considerable threat faced by these students resulting in major health and social issues. Both, good and bad habits are easily picked up and tend to be a norm in later adulthood. These contribute to the already alarming morbidity and mortality prevalence of nation's healthcare. Due to the sensitive nature surrounding the taboo topic of sexuality in Malaysia, adolescents in this country lack knowledge, guidance and even health support services that are related to reproductive health. With their limited knowledge about their bodies and their sexuality, adolescents are vulnerable to sexually transmitted infections e.g. HIV/AIDS, unplanned early childbearing and unsafe abortions (1). Another health hazard that is related to adolescence is road traffic accidents (RTAs). With more than 20 million people killed a year due to road accidents, it causes more death than wars and diseases put together (2). In Malaysia, road traffic accidents are among the ten top causes of death in Malaysia. A Malaysian government fact sheet reports that 56.3% of motorcycle accidents involve adolescent age 16-25 years (3). Undergraduates face increased risk of motor vehicle accidents involvement

due to driving inexperience and impulsiveness. In the United States, 71% of all deaths among youth and adults aged 10–24 years result from four causes: motor vehicle crashes (31%), other unintentional injuries (14%), homicide (15%), and suicide (11%) (4). Another health problem associated with adolescence in Malaysia in drug abuse. In a study conducted in 2002, it is shown that 18.5% of the drug addicts admitted to drug rehabilitation centres in Malaysia age 18-24 years (5). While these problems are very apparent, they are preventable and at present, efforts to reduce the incidences of such problems have been said to be enforced through various social programs. However, we come to question the effectiveness of such programs as the numbers reported may not be reflective of today's society. More so when we disagree to the notion of certain parties that these issues are generally related to adolescence of the lower income group and not of the more metropolitan or educated Malaysian population. We believe that this problem plagues not only of the "less educated" adolescent but affects a large proportion of the students of higher learning, such as college students. However, such data are not available and therefore such a hypothesis cannot be proven or disproven. In this study, we hope to shed some lights on the current situation and trends among tertiary students in the public institution in which this study is carried out.

Methodology

The study was a university-based cross sectional study carried out in the University of Malaya. Included in the study population were undergraduates studying in the university who either stayed inside or outside the campus. A sample of the study population was undertaken. Undergraduates who were long-distant learners and were doing the courses externally were excluded from the study. A traditional definition of undergraduate is employed, that is a student who has freshly graduated from a pre-university program. As such, the vast majority of students are between 18-24 years of age.

Considering the design effect and response rate, a total sample size of 1657 undergraduates was calculated based on the lowest prevalence to estimate a predicted prevalence of 5 % (for prevalence of exposure for intercourse) with a precision of 5% and a confidence level of 95%. The total target population in the year 2007/2008 session was 18018 (6). The sample size was calculated using the program EpiInfo statistical calculator.

The sample was picked following a stratified random sampling method. The sample was stratified to Arts and Science stream according to the nature of their study. Proportional allocation was given to faculties from the Science and Arts streams. The samples from each stream were picked in a random manner so that they equate roughly half of the total sample size. The selected faculties are as follows:

Table 1: *Faculties chosen for the study.*

SCIENCE		**ARTS**	
No.	Name of Faculty	No.	Name of Faculty
1	Computer Science and Information Technology	1	Business and Accountancy
2	Dentistry	2	Economics & Administration
3	Science	3	Law
4	Built Environment	4	Malay studies (Academy)

Data collection was done using a set of questionnaire. With reference to well-tested and standard questions from many surveys carried out to determine the health risk behaviour among youth and undergraduates, such as the CDC Youth Risk Behaviours Survey Year 2007, a set questionnaire were developed to suit local circumstances and settings. Among the areas which were investigated are as follows:

- Demographic background
- Academic background and nature of studies
- Motor Vehicle Accidents (MVA) and road safety prevention precautions.
- Alcohol abuse and tobacco usage.
- Sexual exposure

The questionnaire consisted of 72 questions. The original questionnaire was conceived in English and subsequently translated into the Malay language to facilitate the vast majority of the participants. In Malaysia, the Malay language is the official and national language and understanding the language was a prerequisite for a Malaysian to enter a public university whereas English was learnt as a second language. The questionnaire was designed to protect the students' identity by not disclosing names to encourage anonymousity and voluntary participation.

A pre-testing was carried out and was done on 4 female and 4 male first year biomedicine students. Biomedicine students were chosen as they are not part of the sample to prevent overlapping.

Data collection from the set of questionnaire was self-administered at the respective faculties from January to April of the year 2008. Questionnaires were distributed to willing students after their respective lectures or tutorials. The data is cleaned and edited for inconsistencies prior to analysis. Out of a total of 1728 sets of questionnaires collected, 106 sets were discarded because more than half of the questionnaire was incompletely filled. This leaves the study with 1622 sets of usable questionnaires. Data collected was entered and analyzed using the SPSS program. Relevant data analysis was done using a significant level of 0.05. Due to some missing answers to certain survey questions, the denominator used in percentage computation varies according to the responses

obtained, which was also termed as valid percentage. The response rate was calculated based on the proportion of questionnaire successfully collected relative to the number distributed.

Results

Table 2: *The sociodemographic background of the participants.*

	FREQUENCY	PERCENTAGE (%)
SEX		
Male	420	26.0
Female	1194	74.0
ETHNIC GROUP		
Malay	873	54.039.4
Chinese	636	3.8
Indian	61	1.9
Bumiputera	31	0.9
Others	15	
RELIGION		
Islam	894	55.4
Buddhist	528	32.7
Christian	114	7.1
Hindu	50	3.1
Others	28	1.7
MARITAL STATUS		
Single	1597	98.9
Married	18	1.1
PLACE OF ORIGIN		
Urban	940	58.4
Suburban and Rural	670	41.6
ACCOMODATION		
Residential college	1060	66.3
Own house	149	9.3
Rented house or room	390	24.4

The response rate for the study was 94%. Out of the total sample population of 1622 students, 74.0% was female; a reflection of gender composition in a Malaysian institute of higher learning. The mean age for the sample was 21.3 years (SD= 1.59, range= 26 years).

On ethnic distribution, the sample consisted of 54.0% Malays, 39.4% Chinese, 3.5% Indians, 1.9% Bumiputeras and 0.9% from ethnic group other than stated. A total of 98.5% of the undergraduates were unmarried. Almost 68.4% of the respondents originated from urban areas while the rest of the total respondents were from the suburbs and rural areas. The majority of the participants, 66.3% stayed in residential colleges. Residential colleges are located inside the campus or within walking distances to the university campus. Other respondents either rent a place outside the university, stayed in their own homes or stay with their relatives'. Approximately 49.1% of the respondents were in a course which is Science related and the rest were Arts related.

Motor Vehicle Accident

Car

From the study, 4.7% of the respondents claimed that they had been involved in a car accident in the past 6 months. Another 4.7% of motorcycle riders had involved in an accident in the past 6 months.

Table 3: *Road safety behaviours practiced by respondent drivers.*

Road safety behaviour	Yes						Pearson Chi-Square value	p-value
	Male		Female		Total			
	n	%	n	%	n	%		
Have been involved in a car accident (for the past 6 months)	26	**6.2**	49	**4.1**	76	**4.7**	3.053	<0.08
Have always used indicator when changing lanes	345	**94.8**	755	**96.7**	1100	**96.1**	2.324	0.127
Have always put on seat belt	292	**80.7**	657	**84.6**	949	**83.3**	2.693	0.101
Have always adhere to speed limit when driving	197	**54.0**	504	**64.7**	701	**61.3**	12.050	<0.01
Been inside a car driven by a drunk driver before	110	**27.0**	194	**16.6**	304	**19.3**	20.739	<0.01

It is found that 96.1% of the undergraduates who responded have always used indicator when turning or changing lanes. Asked further about road accidents precautions, 83.3% of the undergraduates have always put on their seat belts when driving. 24.9% of Malay respondents, the highest among the ethnic groups admitted that they did not always put on their safe belt when driving. There were no significant association found between the use of safety belts and the rate of accidents for the past 6 months.

Only 61.2% of the respondents claimed they had always adhered to speed limits when driving. Higher proportion of females 64.7% adhered to speed limit compared to their male counterparts 54.0%.

The overall percentage of respondents who had been inside a car driven by a drunk driver was reported to be 19.3%; males made up a higher percentage 27.0% compared to

female respondents 16.6%. Characteristic of respondents who have driven after the intake of alcohol is shown in Table 4.

It was also found that 7.2% had driven after intake alcohol before. There are more male students (13.7%) who seemed to have driven after intake of alcohol compared to their female counterpart (4.2%). Christians and students who rented their rooms or house also have higher percentage of such practices compared to other groups, 20.0% and 11.2% respectively. It do not seem to be related to the nature of study, that is, whether they are take a course related to science or arts.

Table 4: *Background characteristics of respondents who drive after intake of alcohol.*

Background characteristics	Yes		Pearson Chi-Square value	p-value
	n	%		
Sex				
Male	50	13.7	33.64	<0.01
Female	33	4.2		
Religion				
Islam	8	1.5	63.227	<0.01
Buddha	51	10.9		
Hindu	1	3.1		
Christian	18	20.0		
Ethnicity				
Malay	8	1.5	65.84	<0.01
Chinese	66	11.9		
Indian	2	5.0		
Bumiputera	6	37.5		
Others	1	11.1		
Accomdation				
Residential college	35	5.0	13.41	0.001
Own house	12	9.5		
Rented house/room	35	11.2		
Place of Origin				
Urban	63	8.4	4.28	0.04
Suburban	20	5.1		
Nature of Study				
Science	43	7.0	0.081	0.77
Arts	40	7.4		

Motorcycle

When asked about having rode a motorcycle before, 56.4% has done so, 77.6% consisted of males and 49.0% females. Only 89.1% had always worn helmet when riding in the campus while 89.2% had done so when riding outside the campus. However, of those who used helmet, only a further breakdown of 89.4% of the respondents said they had put on their helmet appropriately including buckling up. About 51.3% reported that they had attempted to wear bright or easily visible shirt when riding at night.

Table 5: *Road safety behaviours practiced by respondent motorcyclist.*

Road safety behaviour	Yes						Pearson Chi-Square value	p-value
	Male		Female		Total			
	n	%	n	%	n	%		
Have rode a motorcycle before	318	**77.6**	573	**49.0**	891	**56.4**	100.9	<0.01
Have been involved in a motorcycle accident (for the past 6 months)	40	**9.5**	35	**2.9**	75	**4.7**	30.476	<0.01
Wears a helmet when riding inside the campus	303	**94.7**	474	**85.9**	777	**89.1**	16.225	<0.01
Wears a helmet when riding outside the campus	298	**92.8**	500	**87.1**	798	**89.2**	6.987	0.08
Wears bright coloured clothes when riding at night	154	**47.2**	315	**53.5**	469	**51.3**	3.272	0.07
Buckle up helmet correctly	260	**87.0**	485	**90.8**	745	**89.4**	3.034	0.08

Tobacco Use

When asked about tobacco usage, about 10.8% of the students had smoked before. Based on gender basis, the prevalence of smoking was found to be 26.6% in males and 5.3% in females. The mean age of their first try was 15 years overall (S.D = 3.94). The mean age for the first try of smoking among male respondents was 14.59 years and 15.78 years among female respondents. At the time the study was carried out, 3.5% of the respondents were still smoking.

The maximum number of cigarette smoked per day was 20 sticks with the mean of 8 sticks per day. Besides, 94.5% of the respondents could correctly name at least one of the risks of smoking and 50.3% of the respondents admitted that they have at least a family member who smoked. 69.8% of the respondents claimed that they were exposed to cigarette smoke before while in campus.

Table 6: *Characteristics of smokers among the undergraduates.*

Smoking behaviour	Yes						Pearson Chi-Square value	p-value
	Male		Female		Total			
	n	%	n	%	n	%		
Have tried smoking before	111	**26.6**	63	**5.3**	174	**10.8**	145.4	<0.01
Still smoking currently	59	**53.2**	18	**28.6**	77	**44.3**	9.844	<0.002
Have tried to quit at least once before	54	**83.1**	16	**66.7**	70	**78.7**	2.811	0.094
Mean age of first puff (years)	14.88 (SD=3.79)		15.78 (SD=3.65)		15.19 (SD=6.71)			
Mean number of cigarette smoked in a day (sticks)	9.10 (SD=6.91)		5.88 (SD=4.85)		8.64 (SD=6.71)			
Knows the risk of smoking	107	**96.4**	59	**93.7**	166	**95.4**	0.691	0.406
Exposed to cigarette smoke in campus	88	**79.3**	41	**65.1**	129	**74.1**	4.221	0.04

Alcohol Consumption and Sexual exposure

On alcohol, 21.3% (n=345) of the respondents had exposure to alcohol. Males comprise 32.9% (n=137) and females 17.5% (n=207) of them. However, only 1.5% (n=25) had drank 3 or more times a week and 79.7% (n=14) of which are males.

The sexual exposure and practice of safe sex was also included in the study. It showed that 6.9% (n=110) had experienced sexual intercourse before. The mean age of their first sexual intercourse was 18.6 years (S.D= 2.99 years) with the mean number of sexual partner of 3 people. The mean age of first intercourse for both males and females were the same with varying standard deviations at 3.13 and 2.88 years for males and females respectively. The proportion of male who had sexual exposure was significantly higher at 15.1% (n=62) compared to females 3.9% (n=46).The study found that 25.9% of the respondents did not practised safe sex. The respondents who took science related courses were more likely to practise safe sex, 82.7% (n=43) compared to those who took arts related subjects, 67.2% (n=43); p=0.058. Of those who practised safe sex, 81.6% (n=80) used condom as one of their contraception and infection transmission prevention method followed by coitus interrupts 29.6 (n=24) and oral contraceptive pill intake 22.8% (n=18).

The study shows that fewer respondents who originated from rural areas practised safe sex compared to their urban counterpart and higher percentage of students had sex if they live further away from the campus.

Table 7: *Practice of safe sex by background characteristics.*

Background characteristics	Yes		Pearson Chi-Square value	p-value
	n	%		
Practise safe sex (condom and/or oral contraceptives)	86	74.1		-
Place of origin				
Urban	57	82.6	6.373	<0.05
Rural	29	61.7		
Accommodation				
Residential college	54	5.2		
Own house	15	10.2	5.461	0.07
Rented house/room	41	10.7		
Alcohol				
Have consumed alcohol before	46	54.8	1.156	0.282
Never consumed alcohol before	38	45.2		

Association with stress level

The respondents of the survey was also asked to rate their own stress level. The scale of stress experienced is a numerical scale ranging from 1 to 5: 1 being the least stressed; 3 being moderately stressed; and 5 being the highly stressful.

Higher percentages of those who are more stress are exposed to health risk behaviours.

Discussion

The sample size of the population was chosen based on the predicted prevalence of sexual exposure of 5%. The value of 5% was picked as an underestimate to the prevalence of sexual exposure found in younger participants in Malaysia which was at 5.4% (1).

There was a higher compliance of safety belt usage among undergraduates where 83.3% admitted to have always put on their safety belts compared to the general public where only 76.6% were seen to have put on their seat belts (7). This result is consistent to the same finding that seat belt compliance was positively associated with educational level, level of enforcement and location as University of Malaya is a public university located at the heart of the capital. As a comparison, the general public of Jakarta

Table 8: *Association of stress level and a few health risk behaviour.*

Stress level	**1 to 3**: Least stressed to moderatedly stressed [N(%)]	**4 and 5**: Between moderately stressed and highly stressful [N(%)]	**Person Chi-Square Value**	**p- values**
Driven after the intake of alcohol	53 (6.0)	30 (11.2)	8.252	0.004
Involved in a car accident in the past 6 months	50 (4.0)	26 (7.6)	8.351	0.005
Ever smoked before	121 (9.5)	53 (15.6)	10.285	0.001
Have ever consumed alcohol	243 (19.2)	102 (30.1)	18.807	<0.010
Had sexual intercourse before	77 (6.1)	33 (9.9)	5.783	0.016

had higher compliance to seat belt use where up to 78% were reported to be using seatbelt (8). However, no data on the percentage of seatbelt use was found for their undergraduates.

The incidence where an undergraduate has been in the car with a drunk driver was considerably lower compared to western countries. The percentage of lifetime exposure found in this study was 19.3% compared to over 28.5% in the United Stated although the study was done over a much shorter time period of only 30 days (3). In the United States, 29.0% of respondents of a study involving college students were reported to have driven after intake of any amount of alcohol. A lower percentage is seen in this study where 7.2% of the respondents in this study admitted to have done it. Although there were few published articles reporting on drunk driving specifically among tertiary students regionally, the percentage of drunk driving is lower compared to the general population reported elsewhere amongst European countries. Locally, it is estimated that 30% of road accidents nationwide are caused by drinking and driving (9). A national study published in the year 2004 found that road traffic accidents is the main contributed for Disability-Adjusted Life Years (DALYs) among males aged 15 to 29 years old amounting to 70,469 or 24.9% of the total. It is the third main cause of DALYS among females of the same age with 9549 or 6.8% of the total. One of the contributing factors to the overall lower prevalence of driving under the influence of alcohol was the composition of the sample population and therefore the population in this area. Malaysia is made up mainly of Muslims who are prohibited from taking alcoholic drinks. However, the prevalence of driving after the intake of alcohol among other races was higher.

Motorcycle riding is a convenient and relatively cheap mode of transportation. It is especially popular among university student. From this study, 56.5% of the undergraduates had ridden a motorcycle before at some point of their life. However, riding a motorcycle also bears a high risk of injury, especially among youth.

The use of helmet was one of the most studied safety behaviours among motorcyclist. It is found in this study that the rate of unprotected motorcycle riding in terms of safety helmet usage were 11% when riding inside the campus and 10.8% when riding outside the campus. These figures were low compared to the rate of unprotected riding obtained from the general public in Malaysia, at a value of 24.2%. As a comparison to Indonesia, where helmet use is also legally mandatory, the percentage of motorcyclists who does not use helmet among the general public there is similar to the percentage found among the undergraduates here at 11% (10). Nevertheless, helmet usage here is much higher compared to less developed countries such as Pakistan where only 56.3% were reported to wear helmets (11).

Moreover, among those who wear a helmet, it is often improperly worn. About 10.5% of those who wore helmet put them on improperly in this study compared to the general Malaysian population at the figure of 21.4% (12). 10.8% (n=174) of the undergraduates had ever smoked before. 26.6% were males and 5.3% were females. The percentage found in this study was lower compared to the general population of the age group 20-24 years which was at 26.4% for those who have ever smoked and 24.7% for those who were currently smoking for the same age group. The percentage found in this study was also lower compared to the percentage of smokers in the general population who have attained tertiary education which was at 14.2% (13).

Mean age of the respondentwhen thet first tried smoking was 15 years. This age was considerably younger if it was to be compared with other countries. In Indonesia, the average age of first puff was 17.4 years in the year 2004 (14). The maximum number of cigarette smoked per day was 20 sticks with the mean of 7 sticks per day which was also lower compared to the general population at 12.1 sticks per day (13).

The prevalence of sexual exposure found from this study was 6.9% overall; 15.1% among males and 3.9% among females. These figures were still lower than other Asian countries such as Korea which reports 23% of males and 10% of female university students had sexual experience (15). Another local study done to determine the prevalence of sexual prevalence among 4500 secondary school students found the prevalence to be 5.4% (1). The percentage adolescent does not use any contraception among undergraduates (25.9%) was also lower compared to a neighbouring country which reports 48% of unmarried

females never use any contraception (16). Despite this, a lack of safe sex practise in Malaysia was still a worrying trend. This is in view of the number of women and young girls being infected with HIV had risen from 4% in 1995 to 18% in 2009 (17). It was also stated that 25% to 40% of them had contracted the disease from their husbands or boyfriends. The Malaysian government recently announced that sex education will be implemented nationwide after the response to the pilot project were encouraging in providing knowledge and awareness in sexuality, pregnancy prevention, unsafe abortion, abandoned babies and sexually-transmitted disease. The mean age of first sexual intercourse in this study was 18.64 years for both males and females, compared to a developmentally equivalent South East Asian country, the Philippines which were 18 years for males and 18.3 years for females (18). However, it was lower that national data which reported 24.8 years and 22.8 years in males and females respectively among the general population (13).

The study which was carried out presented with several limitations. First, the data collected may not truly reflect the undergraduates as those who participated in the study were voluntary. Secondly, there may be under or over-reporting as in many self-administered questionnaires. Although, steps were taken to minimize reporting error, under-reporting or over-reporting cannot be ruled out. Steps to minimize reporting error included ensuring anonymity through steps such as recruiting participants on voluntary basis, having no questions on the first and last page of the questionnaire, allowing as much time as needed for questionnaire completion and collecting the completed questionnaire as soon as completed. One presenting problem in carrying out the study is that not all the students finish the questionnaires at the same time, reducing privacy towards the end of the session. Due to lack of facilities and expected reduction in the turn out, the session could be carried out in separate cubicles. Cigarette smoking was the only measure of nicotine intake assessed in this study. Other forms of tobacco use (skin patches) were not measured in this study albeit not popular.

On the other hand, the study is only conducted in one public university in Kuala Lumpur; data collected from the study may not reflect the general situation in the whole country as different campuses may have different student make-up and local settings. This study may not represent the true situation in private institute of higher learning due to differences in student composition, rules and other local settings.

Conclusion and Recommendation

Our study demonstrates that undergraduates take unnecessary risks on the road as reflected by the low practices of road safety behaviour. Safety belt usage was still considerably low even among university students despite the fact that a law has been put in place for many years. Driving consuming alcohol was not as rampant as it was in the West but still pose a dangerous practice. Data on sexual behaviour from around the Asian region was not readily available but from places where it was available, the prevalence of university students engaging in sexual activities was much higher than in Malaysia than had been anticipated.

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OPTIMIZATION AND VALIDATION OF A CELL-BASED TYROSINASE ASSAY FOR SCREENING OF TYROSINASE INHIBITORS

Chan YY, Kim KH, Cheah SH

Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur

Correspondence:

Chan Ying Ying
Research Officer
Institute for Public Health
Ministry of Health Malaysia,
Jalan Bangsar, 50590 Kuala Lumpur.
Phone : 03-2297 9400
Fax : 03-2282 3114
E-mail : chan.yy@moh.gov.my

ABSTRACT

Tyrosinase is a key enzyme that catalyzes melanogenesis in human skin. It oxidizes tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to dopachrome, which further polymerizes to melanin pigments. Therefore finding an effective tyrosinase inhibitor, either from synthetic or natural sources, is not only useful as skin whitening agents in cosmetic application, but also beneficial in treating melanin-related disorders. The present study reports of the optimized and validated results of a cell-based tyrosinase assay using B16F10 murine melanoma cell line, which produces melanin pigments and has been used extensively in antimelanogenesis studies. The optimization studies involved 3 parameters (1) optimal seeding cell number per well for total protein extraction; (2) optimal dopachrome formation from enzymatic reaction between total protein (tyrosinase source) and L-DOPA (substrate); and (3) optimal incubation period after the addition of substrate. The present study demonstrates that using seeding cell number of 2×10^5 cells/well, total protein of 40 µg, L-Dopa of 5 mM,and at an incubation period of 1 hour at 37°C provided the optimal response on cultured melanoma cells. Kojic acid, a standard tyrosinase inhibitor, was used as a positive control in the optimized cell-based tyrosinase assay to validate the usefulness of the assay. CONCLUSION: The use of the mentioned protocol is sensitive to determine changes in melanoma cells as the result of tyrosine inhibitors.

Keywords: *Tyrosinase, tyrosinase inhibitors, melanogenesis, melanin, whitening*

Introduction

Melanin production, or melanogenesis, is principally responsible for skin and hair colours, and plays an important defensive role against the harmful effects of ultraviolet radiation of sunlight. However, overproduction and accumulation of melanin result in various skin dermatological disorders including melasma, freckles, age spots, and sites of actinic damage or other hyperpigmentations (1, 2). Melanin is produced normally by melanocytes and, to a greater extent, by melanoma cells. In mammals, melanin is synthesized in the melanosomes of melanocytes that contain tyrosinase, which plays a key role in melanogenesis. Tyrosinase, a copper-containing binuclear enzyme, catalyzes two rate-limiting steps of melanogenesis: that is, the hydroxylation of L-tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to dopaquinone (3). For this reason, tyrosinase is an attractive target in the search for various kinds of whitening agents. Agents that inhibit tyrosinase activity have the potential to be antimelanogenesis agents and thus promote depigmentation, such as hydroquinone and kojic acid (4).

Recently, safe and effective tyrosinase inhibitors have become available which can lead to potential applications in preventing pigmentation disorders and other melanin-related health problems in patients. Tyrosinase inhibitors are also important in cosmetic applications to produce the skin

whitening effects (5). The whitening potential of a compound is commonly measured by inhibiting mushroom tyrosinase in cell-free systems (6, 7). However, it does not adequately represent the whitening effects since it does not account for cellular uptake of the test samples. Several studies have indicated that many plant extracts shows inhibitory activity against mushroom tyrosinase *in vitro* but did not reduce the pigmentation activity in cells (8). Conversely, other compounds tested on cellular tyrosinase in cultured melanocytes did not produce similar results although detected as having tyrosine inhibitory activites(9, 10). Furthermore, it should be noted that some major differences exist between the mushroom tyrosinase and the mammalian tyrosinase, causing many different effects and may result in untoward symptoms in patients (11). Therefore, in order to determine a safe and effective skin whitening agents from natural sources, a reliable, quick and easy method and materials would be needs. In the present paper we described a novel method that led to the development of an optimized cell-based tyrosinase assay using B16F10 murine melanoma cells. B16F10 murine melanoma cells have been widely used to elucidate the regulatory mechanisms of melanogenesis and pigment cell proliferation and thus would serve as a reliable indicator of the effects of the tested material on potential human skin cells.

Materials and Methods

Cell culture

B16F10 murine melanoma cells (CRL-6475) were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 1% (v/v) penicillin/streptomycin (100 units/ml), and incubated at 37°C under 5% CO_2 atmosphere. Cells used for experiments were maintained in lower cell passage number (less than 10 passages) to avoid batch-to-batch variations in tyrosinase activity measurement. B16F10 melanoma cells were seeded into 6-well plates for the cell-based tyrosinase assay.

Optimization studies

The cell-based tyrosinase assay described here was designed to incubate the cells with test sample for 72 h at 37°C. The assay was a modification of a previously described method (12, 13) and involved optimizations of some parameters as stated below:

(i) Determination of the optimal seeding cell number per well that yielded sufficient protein containing tyrosinase for the assay;
(ii) Determination of the amount of total protein and concentration of L-DOPA (substrate) required for optimal dopachrome formation; and
(iii) Determination of optimal incubation time for dopachrome formation after the addition of substrate.

For determination of optimal seeding cell number per well, cells were plated in 6-well plates at an increasing seeding cell number ($1 \times 10^5 – 5 \times 10^5$ cells/well) in 3 ml of culture medium without test samples. After an overnight incubation, the medium of each well was replaced with fresh medium. The cells were cultured at 37°C for another 72 h. After 3 days incubation, the cells were harvested and lysed with M-PER mammalian protein extraction reagent (Pierce). The cell lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C and the total protein content was quantified by using a protein assay kit (Bio-Rad). The cell-extracted protein or total protein was used as the source of crude tyrosinase for the tyrosinase assay. Total protein extracted per well increased proportional to the number of cells seeded per well. Seeding cell number that reached confluency on day 5 and gave an adequate amount of total protein (200-400 µg/well) for the tyrosinase assay was determined as the optimal seeding cell number.

After cell lysis on day 5, protein concentrations were adjusted to a range of 20-100 µg/100 µl/well with 0.1 M sodium phosphate buffer (pH 6.8) in a 96-well plate. Then, 100 µl of freshly prepared L-DOPA solution at different concentrations (2.5, 5.0 and 10.0 mM) were added for dopachrome formation. The relationship between total protein and concentration of L-DOPA for dopachrome formation was observed.

The reaction mixture (200 µl/well) consisted cell-extracted protein and L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8) was added into wells of a 96-well plate in triplicates. The plate was incubated at 37°C and absorbance was measured at 475 nm for a time course of up to 4 hours in order to determine the optimal incubation period for measurement of tyrosinase activity. The absorbance of the dopachrome formation increases over incubation time. The changes in absorbance values were monitored every 10 min for the first two hours and every 30 min for the subsequent hours up to a total incubation period of 4 hours.

Kojic acid, a known tyrosinase inhibitor, was used as a positive standard to confirm the efficacy of the optimized cell-based tyrosinase assay. Using the optimized protocol, after 3 days incubation of kojic acid at increasing concentrations (100, 250 and 500 µg/ml) in B16F10 melanoma cells, the tyrosinase inhibitory activity was measured. The values obtained were compared to the data of inhibitory activity of kojic acid reported in other papers. Values are expressed as mean ± standard deviation (SD) of at least 3 independent determinations.

Results and Discussion

A radio-enzymatic method for the measurement of tyrosinase activity was first introduced by Pomerantz (14) and has been extensively employed in studies of melanin synthesis, using tissue homogenates or cultured melanoma cells as substrates. In this study, the tyrosinase assay was applied to a cell culture system using B16F10 melanoma cells for antimelanogenesis study.

According to our optimization results, the amount of total protein extracted from the cells increased proportionally to the initial seeding number of cells per well up to a stage where the cells wver beyond confluent (data not shown). We have selected 2×10^5 cells/well as the optimal seeding cell number because the cells reached confluency after the incubation periods and produced sufficient protein content for the cell-based tyrosinase assay (triplicates). Dopachrome is formed from the enzymatic reaction between the total protein (tyrosinase source) and L-DOPA (substrate). The amount of dopachrome produced depends on the amount of total protein and the concentration of L-DOPA used in the enzymatic reaction. Figure 1 shows that increasing amount of total protein (20-100 µg) reacts maximally with 5 mM of L-DOPA and gives an increasing absorbance values at 475 nm. It was observed that 5 mM of L-DOPA produced better readings of absorbance values compared to 2.5 mM of L-DOPA. More cells are needed for the assay if higher amount of total protein is chosen for the enzymatic reaction. Therefore, based on the optimal seeding cell number that we have selected and the amount of total protein that can be extracted from the cells, the minimum acceptable dopachrome formation was at 40 µg of protein and 5 mM of L-DOPA. These levels of protein and L-DOPA were adopted for optimal dopachrome formation which produced acceptable absorbance value at 475 nm. A time course study of cellular tyrosinase activity in B16F10 cells up to four hours demonstrated that the dopachrome formation inces overduring incun time at 37°C resulting in an increasing absorbance value at 475 nm (Figure 2). We have adopted one hour as the optimum incubation period for end-point measurement of tyrosinase activity due to the rate of cellular tyrosinase activity was rapid and linear in the first hour and produced acceptable absorbance value at 475 nm.

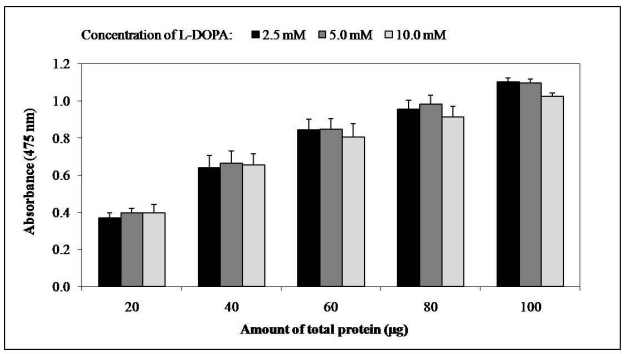

Figure 1: The relationship between the total protein and the concentration of L-DOPA for dopachrome formation in cell-based tyrosinase assay. Each value represents the mean ± SD (n=3).

To verify the usefulness of the optimized assay, kojic acid (a standard tyrosinase inhibitor) was tested as a positive control. Kojic acid at concentrations of 100, 250 and 500 µg/ml showed dose-dependant cellular tyrosinase inhibitory activity of 15.44%, 31.23% and 41.37% in B16F10 cells respectively. The inhibitory activity of kojic acid measured by this optimized assay is comparable to the tyrosinase inhibitory activity of kojic acid reported by Im *et al.* (15) on the same cell line, B16F10 melanoma cells. This finding indicates that our optimized cell-based tyrosinase assay, is useful and valid as compared to other reported studies, in which kojic acid was used as a positive control.

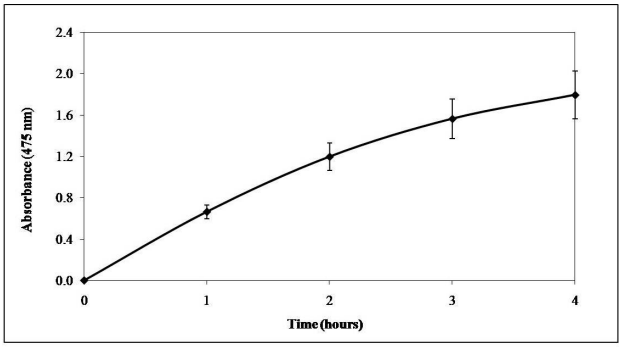

Figure 2: Time course study of cellular tyrosinase activity using 40 µg of protein and 5 mM of L-DOPA. Each value represents the mean ± SD (n=3).

Conclusion

The development of an optimized and standardized cell-based tyrosinase assay using B16F10 melanoma cells may provide useful information to researchers who are using the same cell line for tyrosinase assay. The optimized cell-based tyrosinase assay is highly reproducible, sensitive and offers a relevant screening tool to search for more effective tyrosinase inhibitors from natural sources such as plant extracts.

Acknowledgement

This study was supported by the postgraduate research grant from the University of Malaya (PS280/2008A) and Fundamental Research Grant Scheme (FP021/2008C) from the Ministry of Higher Education.

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Discussion with Reviewers

Reviewer: The authors claimed that they have developed an optimized assay for tyrosinase. However, a comparison between their final results and those reported in reference (12, 13) shows little difference.

Authors: Optimization of cell-based assay is an important preliminary step to determine optimized condition of the assay for providing relavant results in a laboratory. Current assay was developed and optimized specifically for tyrosinase derived from B16F10 melanoma cells (a subline of B16 melanoma that is highly metastatic). The slight difference in optimization results of our study compared to other studies (Reference 12 and 13) is reasonable as this could be due to the use of different cell line as source of tyrosinase enzyme (reference 12 – use HEMn cells; reference 13 – use B16 melanoma cells) and different laboratory settings.

Reviewer: In the paper, the authors reported the 'optimized conditions' as total protein – 40 µg, L-Dopa – 5 mM; incubation period – 1 hour.

Authors: Yes. Our optimized key assay parameters are: total protein – 40 µg, L-Dopa – 5 mM; incubation period – 1 hour.

Reviewer: In reference 12, the conditions stated are: protein --40µg, L-Dopa 2.5mM, incubation period -1 hour.

There is essentially no difference between data reported by the authors and reference 12, the reason is, of course, that authors of reference 12 had already 'optimized' the method.

Authors: In response to reviewer's comments 3 & 4, the slight difference between the concentration of substrate (L-Dopa) used in our study (5.0mM) and those reported in reference 12 (2.5mM) is reasonable due to the use of different cell line (source of tyrosinase enzyme) and different laboratory settings. At 40µg of total protein, we observed that 5.0mM of L-Dopa reacts maximally and produces better readings of absorbance values compared to 2.5mM of L-Dopa.

Reviewer: The authors claimed that the optimum L-Dopa concentration was 5 mM, but from the data presented in the figure provided, statistically there is no difference between the readings of 5 mM and 2.5 mM. In fact, for almost all protein concentrationa, there is no difference in reading whether L-Dopa concentration is 2.5, 5 or 10mM, and the reason is obvious, all this concentrations are above the K_m of the substrate, and when one measures rate of enzyme catalyzed reaction at substrate concentration >> K_m (saturation), varying the substrate concentration will not alter the rate of reaction.

Authors: In our study, the amount of tyrosinase enzyme present in the cell-extracted protein or the activity of the enzyme itself is the limiting factor, and not the amount of substrate (L-Dopa) available. This means that the concentration of substrate must be high enough to ensure that the enzyme is acting at V_{max}. In practice, it is usual to use a concentration of substrate higher than the K_m in order to determine the activity of an enzyme in sample. It was preferable to use 5.0mM of L-Dopa as it reacts maximally at 40µg of total protein (source of tyrosinase enzyme) and produces better readings of absorbance values compared to 2.5mM of L-Dopa.

Reviewer: I must say that what the authors reported are essentially preliminary work before the study of any action of enzyme –ie, setting the enzyme activity determination conditions, and works of this nature are not acceptable for publication. It is just an essential step in study of any enzymatic reaction.

Authors: Preliminary optimization of cell-based tyrosinase assay is essential before the study of tyrosinase inhibitory activity for screening of tyrosinase inhibitors. We verified the usefulness of the optimized assay by testing with a positive control – kojic acid (a standard tyrosinase inhibitor). We reported the development of an optimized and standardized cell-based tyrosinase assay using B16F10 melanoma cells which may provide useful information to researchers who are using the same cell line for tyrosinase assay.

DETERMINING THE EFFECTIVENESS OF THE DIFFERENT PREPARATION PROTOCOLS FOR PLATELET-RICH PLASMA (PRP) IN YIELDING HIGHER CONCENTRATIONS OF PLATELETS

Kavitha G, Sangeetha VN, Shani S, Murali MR, Raja EA, Rukmanikanthan S and David C

Tissue Engineering Group, Department of Orthopaedic Surgery, NOCERAL,
Faculty of Medicine, University of Malaya, Kuala Lumpur.

Correspondence:

Sangeetha Vasudevaraj Naveen
Tissue Engineering Group (TEG), National Orthopaedic Centre of Excellence in Research and Learning (NOCERAL),
Department of Orthopaedic, Faculty of Medicine,
University of Malaya,
50603 Lembah Pantai,
Kuala Lumpur, Malaysia.
Tel: +603-7967 7543, +6012-532 7289, Fax: +603-7949 4642
E-mail: sangeethanaveen@um.edu.my

ABSTRACT

INTRODUCTION:
Despite the various methods described in producing platelet-rich plasma (PRP), it is well established that this biological product in its many preparations have been proven to enhance wound healing. However, very little have been known about the efficacy of these methods hence there is a lack of evidence in the superiority of one method over another. Thus, a study was conducted to compare these different protocols to determine which produces the highest concentration of platelets.

METHODS:
Peripheral blood was obtained from 24 healthy volunteers. Four different protocols using similar 2 step centrifugation methods of preparing PRP were applied to an equal number of samples in this study. Platelet counts were performed on whole blood (without processing), PRP preparations and platelet-poor plasma (PPP).

RESULTS:
All protocols produced higher amounts of platelet concentrates in PRP preparations than plasma. However, centrifugation at 150g for 10 minutes followed by another at 450g at 10 minutes produces significantly higher amount of platelets concentration ($p<0.05$).

CONCLUSION:
Optimizing the protocols to produce PRP appears to be important in obtaining a maximal yield of platelet concentrate. Here the protocol described has shown to provide significant concentration yield over all others.

Keywords**:** *platelet-rich-plasma, growth factors, centrifugal forces*

Introduction

Platelet rich plasma (PRP) is a highly concentrated form of autogenous platelets, providing a rich and readily obtainable source of a diverse group of growth factors(1). Its importance is related to the large variety of growth factors involved in healing that are physiologically contained in platelet α-granules (2). Although the role of platelet concentrates in treating various haematological conditions and diseases have been well established, their roles in wound healing have only been recently described. PRP mainly consist of platelets and several growth factors which includes (but not limited to) platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), endothelial growth factor (EGF) and insulin-like growth factor (IGF). These growth factors are released from activated platelet to initiate and modulate wound healing in both soft and hard tissues. Growth factors found in higher concentrations are thought to help in accelerating and enhance wound healing through tissue repair mechanisms such as chemotaxis,

cell proliferation, angiogenesis, extracellular matrix deposition and remodelling (3, 4). PRP which contains high concentrations of growth factors, is used for various applications mainly for wound healing that may shorten the healing period. Besides, PRP are widely used in oral, maxillofacial surgery, tendon repairs, muscle therapy and many more.

Based on our literature review, there are several ways to prepare platelet concentrate (PRP), all of which promising higher platelet concentrations. However, we have found there has been no reference to a study which makes a side to side comparison analyses on these various methods and thus provide no evidence of the effectiveness of one particular method over the other. Thus, a study was conducted to compare the different methods of preparing PRP and to determine which method best produces the highest platelet concentrate. In addition, this study will also reconfirm the reliability of different preparation of PRP as claimed in the different literatures.

Methods

PRP is prepared via a 2-step centrifugation procedure using venous blood. The centrifuge speed, the amount of blood collected, the type of equipment used for collection of blood differs according to the type of preparations described by the various authors. Blood was obtained from 24 subjects who have no previous known medical illness and appeared well on the day where the procedure was performed.

A venepuncture was performed by a phlebotomist to obtain the peripheral blood. 10 ml of blood was aspirated with a 21 G needle and was mixed with 18 mg of anticoagulant K2 potassium salt of ethyleneediaminetetraacetic acid (EDTA) to avoid coagulation. 0.2 ml of blood was taken and placed in a sterile tube where whole blood count was performed. PRP was isolated from whole blood at different centrifugal force at different time period which results in a 0.5-1ml "buffy-coat" extract. The layer was then separated centrifuged again to obtain a two-part plasma: the upper layer which is the poor-platelet plasma (PPP) and the lower layer known as PRP. The PRP and PPP were then separated and place in sterile tubes. A series of platelet count was performed using the Sysmex XT-1800 machine.

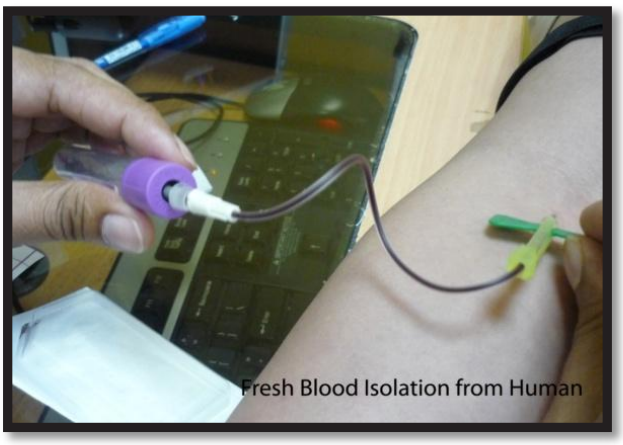
Statistical Analysis

The data obtained were analyzed using statistical software (SPSS for Windows; PASW Statistics version 18). The number of platelets in whole blood, the number of platelets in PRP and the number of platelets in PPP were compared with different methods of preparations. Non-parametric test ($p<0.05$) were employed in this study.

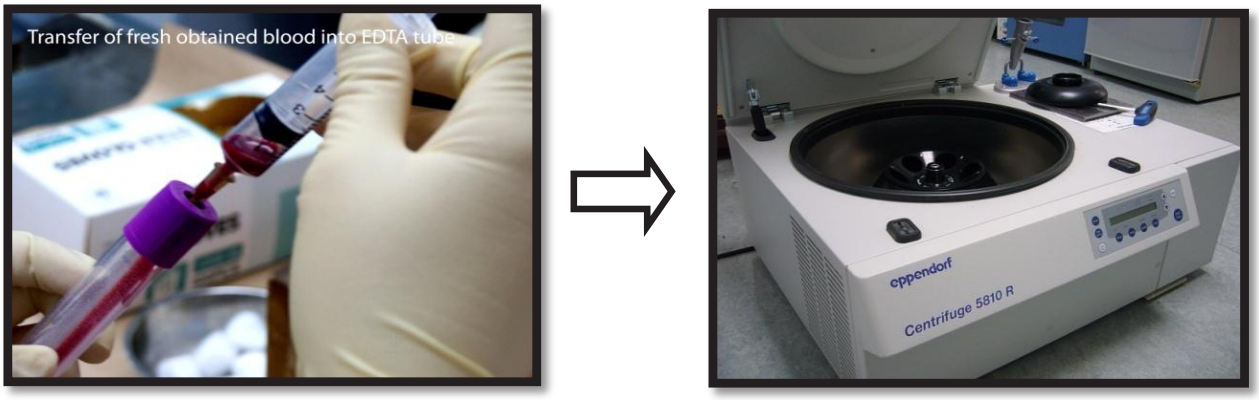
Table 1: The different centrifugation speed, period of time and temperature taken for the comparison of which best produces the highest platelet counts.

Method	1st Centrifugation speed	Time (Minutes)	Temperature (°C)	2nd Centrifugation Speed	Time (minutes)	Temperature (°C)
1	2000 g	30	20	250 g	20	20
2	150 g	10	20	450	10	20
3	215 g	10	20	865	10	20
4	1125	10	20	300	25	20

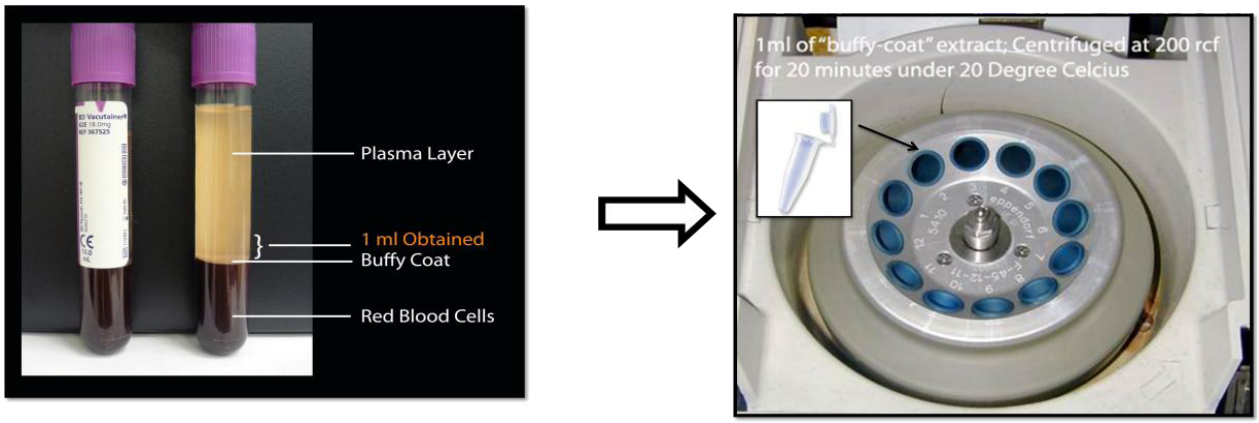
Isolation of blood through venipuncture.



Obtained blood was transferred into the 10 ml EDTA tube (anti-coagulation) and centrifuged at different speed as stated in Table 1



Three layers were formed (Top layer: Plasma, Middle layer: Buffy Coat, Lowest Layer: Red Blood Cells). 1 ml of plasma layer right above the Buffy coat was obtained and centrifuged again.



Two layers were formed: Lower layer Platelet-Poor Plasma (PPP) and Upper Layer of Platelet-Rich Plasma (PRP)

Flow Chart 1: Shows the two centrifugation technique that was performed. Data was attained for whole blood counts, PRP counts and PPP counts.

Results

PRP was prepared using different centrifugal forces which were described by previous authors (5-7). Regardless of the method used, the numbers of platelets in PRP were of higher value as compared to the number of platelets in whole blood. Figure 1 to 4 is a summary comparing the values attained based on platelet counts from whole blood and PRP. All four methods proved that platelet counts for PRP is of 2-5 folds higher as compared to the platelet counts in whole blood.

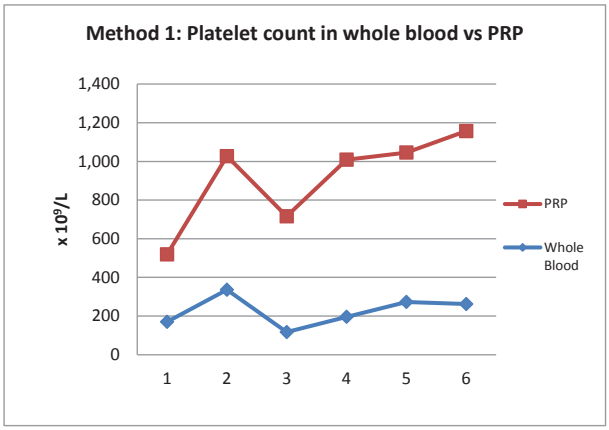


Figure 1: The graph above shows the platelet count in whole blood and PRP obtained by using Method 1

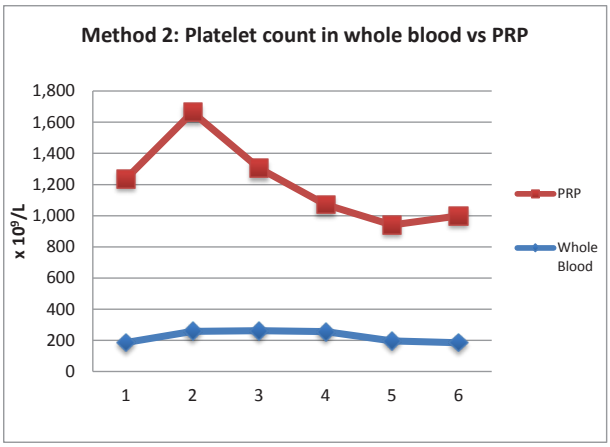


Figure 2: The graph above shows the platelet count in whole blood and PRP obtained by using Method 2

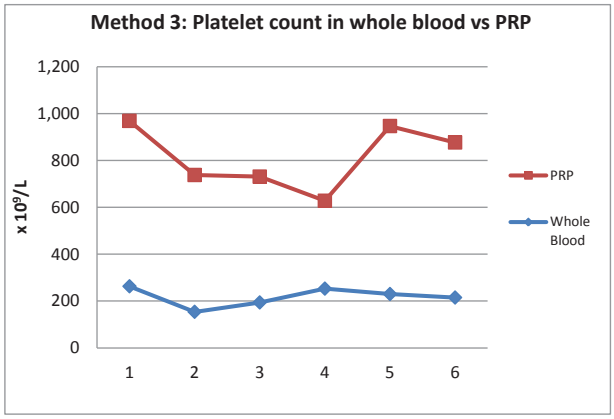


Figure 3: The graph above shows the platelet count in whole blood and PRP obtained by using Method 3

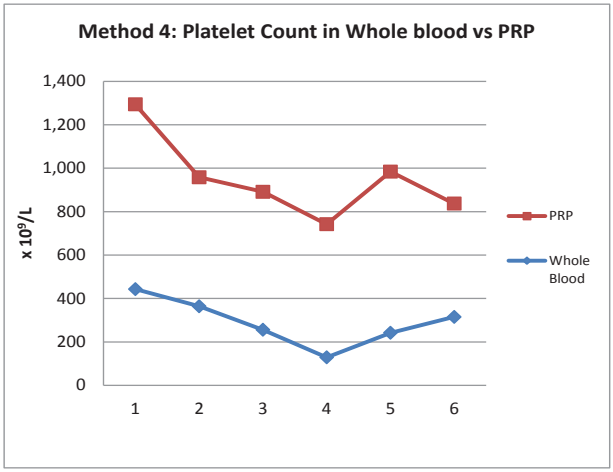


Figure 4: The graph above shows the platelet count in whole blood and PRP obtained by using Method 4

There were 11 females and 13 male donors in this study. The mean of the age for the subjects who participated for this study lies within the range of 24 to 29. There were no significant differences between the mean age and the values attained for both sexes (Table 2 and 3). Based on statistical analysis, it was found that the distribution of platelet counts in whole blood in method 1,2,3 and 4 were not significantly different ($p < 0.05$). It was also found that that the distribution of platelets in PRP attained using methods 1, 2, 3 and 4 were of normal distribution.

Table 2: Mean Values for Platelets in Whole Blood, PRP and PPP.

Number of Subjects				Mean no of Platelets (x 10^9/L)			
Method	Males	Female	Age	WB	PRP	PPP	p-value
1	4	2	28.83	256.67	686.67	24.50	0.135
2	2	4	24.67	223.83	979.17	59.17	0.02
3	3	3	24.83	218.17	596.83	35.00	0.135
4	4	2	24.50	291.50	659.50	212.60	0.007

*p-value denoted is by comparing p-values of PRP and PPP by using Mann-Whitney U test.

It is found that the mean number of platelet counts for PRP obtained by following method 2 produces the highest platelet count (Kruskall-wallis: $p < 0.05$). All other methods only produced between 2.2 to 2.75 fold increase in platelet counts (table 3).

Table 3: Data analysed on the number of folds comparing whole blood with PRP.

Mean no of Platelets (x 10^9/L)				
Methods	WB	PRP	Number of folds	p-value
1	256.67	686.67	2.67	
2	223.83	979.17	4.37	0.87
3	218.17	596.83	2.74	
4	291.50	659.50	2.26	

*p-value denoted is by comparing p-values of PRP with different methods by using post hoc – Tamhane's T2 test.

Discussion

The realization in the vast potential of PRP in clinical applications have made the use of this simple to produce biological product for maxillofacial surgery, foot and ankle surgery (8), osteal defects repair (9, 10), muscle and tendon therapy (11-13) and many more, a dispensible alternative to current more elegant and expensive methods of treatment including stem cell therapies. However, before the use of PRP can be effective, several issues needs to be addressed which includes identifying the most important method to produce the most effective therapeutic concentration possible. The type of anti-coagulant, centrifugal speeds, the amount and the type of growth factors existing in PRP, the number of platelets in the donor's blood and PRP itself as well as the clinical applications of PRP all play an important role in determining the maximal yield of platelet concentrate and therefore must be carefully considered before being applied into clinical applications (14-18). To our knowledge, we found that there were many methods to preparing PRP but no similar comparative study as to the one presented in the present report have been described previously. This study demonstrates the importance of determining optimization methods and standard practices which will eventually lead to the best outcome possible of PRP products.

Further determining the highest possible concentration using various methods of preparations, it is also noteworthy to determine the concentrations of platelets required for wound healing or other applications to be succesful. Haynesworth et al demonstrated that the proliferation of adult mesenchymal stem cells and their differentiation were directly related to the platelet concentration. They showed a dose-response curve, which indicated that, to produce a sufficient cellular response to platelet concentrations, concentration of approximately 4 to 5 fold increase of platelet count as compared to baseline platelet count would be required. It was therefore important to note, that if this study had made its way into clinical applications, method 2 would have been the only method which would have produced a significant clinical outcome while the other methods would prove to be sub-optimal. As most individuals have a baseline platelet count of 200,000 ± 75,000/µL, a PRP platelet count of 1 million/ µL as measured in the standard 6-ml aliquot has become the benchmark for "therapeutic PRP".

Based on the mean platelet counts, the highest in mean number of platelets concentration in PRP was produced by applying method 2 which was 979.17 x 10^9/L. The highest mean number of platelet in PRP is followed by method 1, 4 and 3 (in a descending order) which falls within the range of 500 to 700 x 10^9/L. In reference to the study conducted by Marx et al, other than method 2, all other methods would not be beneficial for patients receiving ineffective concentration of PRP. Due to this factor, FDA cleared devices (concentrates platelets) must consistently achieve this therapeutic levels of platelet concentration and thus growth factor release, in order to be registered as a therapeutic device (17, 18).

As the objective of this study was to compare the different techniques in order to attain the best or the highest platelet count in PRP, the changing variable is the centrifugal forces. Method 1 and method 4 uses "hard spin" for the first centrifugation and "soft spin" for the second centrifugation. Whereas, Method 2 and 3 starts off with soft spin for the first centrifugation and then followed by hard spin. Based on the values attained in this study, it was found that by applying method 2 produces the highest mean number of platelets in PRP, it can be said that, in order to obtain highest number of platelet concentrate, soft spin can be a better option. But this contradicts as Method 1, whereby we used hard spin and then followed by soft spin and this produces highest mean number of platelets as compared to method 3 and 4. There are several articles published that uses hard spin and then soft spin for the second centrifugation and documented success rate in obtaining PRP with high number of platelets (3, 19, 20). Thus, it is unclear and undefined whether the hard spin or the soft spin as the centrifugation force or vice versa for the second centrifugation force as far as obtaining highest number of platelets in PRP is concerned. Also, further studies should be conducted looking into the platelet function based on these using soft spin and hard spin as well as looking into these platelet function in terms of *in vivo* trials.

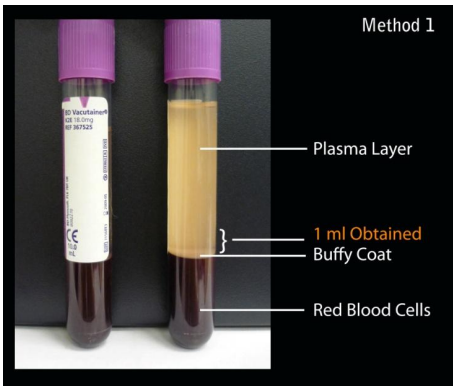


Figure 5: Images taken after the first centrifugation using method 1

Several limitations were identified in this study and were carefully addressed where possible. One of which was the difficulty in distinguishing the plasma layer in our PRP preparations. Based on the images above, it was found that method number 1 shows a clear layer of plasma, buffy coat as well as red blood cells. However, in other methods, it was found to be difficult to delineate the exact PRP layer which lies directly above the buffy coat (21, 22). Furthermore, when it comes to the preparation of platelet, it was difficult to isolate the plasma layer right above the buffy coat running the risk of contaminating the PRP with red blood cells. It therefore appears that the method of sampling PRP in all methods is more of an art rather than pure science and therefore a more amicable and standardized approach to extract the exact layer of platelet concentration must be developed if a more replicable study is to be produced. In addition, although method 2 produced the highest mean number of platelets in PRP, the number of functional platelets remains an unanswered question. Thus, further studies to test the function of PRP in *in vivo* trials must be considered.

Conclusion

The proposed protocol that is used in method 2 which involved centrifugation at 150g for 10 minutes followed by another at 450g at 10 minutes has shown to produce the highest mean number of platelets concentrations in PRP.

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LIST OF REVIEWERS FOR VOLUME 14, ISSUE 2, 2011

Associate Prof. Dr. N. Veera Sekaran A/L V. Nadarajan
Department of Pathology,
Faculty of Medicine, University of Malaya, Kuala Lumpur

Prof. Dr. Tan Nget Hong
Department of Molecular Medicine,
Faculty of Medicine, University of Malaya, Kuala Lumpur

Prof Dr. Francis I. Achike
Clinical Skills, Pharmacology and Therapeutics,
Clinical Sciences Section,
International Medical University, Bukit Jalil, Kuala Lumpur

Prof. Dr. Low Wah Yun
Faculty of Medicine Dean's Office,
Faculty of Medicine, University of Malaya, Kuala Lumpur