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EDITORIAL

NEUROSCIENCES IN UNIVERSITY SAINS MALAYSIA; THE WAY TO GO FORWARD IN MALAYSIA WITH VISION 2020

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Key words *: Neurosciences, Malaysia, Strategy, Developing Country.*

Introduction

The growth of neurosciences in Malaysia is unlike other Asian countries like Singapore, South Korea, China and Japan (1) where a rate of 6 to 20% is expected per annum.

A survey of Googles [www.google.com] indicated that there were only 621, 000 hits concerning scientific neuroscience publications from Malaysia of which 161 were from Universiti Sains Malaysia. Hits on publications on neurology were 345,000 and 115,000 for neurosurgery respectively when these two key words were associated with Malaysia. This is only 20% of what is published by Australia a developed country with a population nearly similar to Malaysia.

National Strategies

The government of Malaysia has started it's focus on biotechnology concentrating on agrobiotechnology as well as on human biotechnology since the launch of the National Biotechnology Policy recently in April 2005. This was done to direct Malaysian scientists and entrepreneurs to achieve the objectives set up by the Malaysian Biotech Corporation (MBC) hopefully by 2020. Currently there are 20 Malaysian scientists per 100,000 labour force and this is expected to grow at 3-6% per year. There are 5,200 Malaysian scientists in the United States of America (USA) and 1, 567 scientists in Australia respectively (2) of whom at least 1% are involved in the neurosciences.

Recently, publications have surfaced in one international journal examining the impact of biotechnological sciences especially the neurosciences in Malaysia. Interested Malaysian neuroscientists overseas were invited to collaborate at local or international level to improve research and development in Malaysia (3,4). The establishment of a national institute in the neurosciences was even suggested by a Malaysian neuroscientist based in Japan (4). Unfortunately, the number of neuroscientists in Malaysia are small and diversed. The number of fundamental and applied neuroscientist registred with International Brain Research Organization (IBRO) are 11 [www.ibro.com] of whom less than five are from Universiti Sains Malaysia (USM)

USM Strategies

USM has set upon producing human resources in this field when it initiated the Master of Science (Neurosciences) and PhD by research in the field of neurosciences in 2002. This came after USM's five years experience in conducting the Master's of Surgery (Neurosurgery) and the establishment of the Department of Neurosciences in the School of Medical Sciences in 2004. An advanced postgraduate Masters of Neurology degree programme is being proposed for the 9th Malaysian Plan, beginning 2007.

Unfortunately, having a respectable structure and resources without the scientists interested in brain sciences can dampen research in this field. The Right Honorable Vice Chancellor of the Universiti Sains Malaysia Dato' Prof. Dzulkifli Razak with his far sightedness did the right move by initiating a Brain Research and Information Network Cluster exactly a year ago [www.brainetwork-usm.org] where different workshops on different neuroscience research themes were organized. This network consists of a group of 80 researchers ranging from the social sciences to the clinical sciences, from all three campuses, networked together to initiate diverse research proposal with the assistance of the Research Creativity and Management Office of this university [www.usm.my/r&d].

This neuroscience group plans to be united

under a common roof where a proposal for a Brain Sciences Institute has been forwarded to the Economic Planning Unit. It has also thus far initiated collaboration with the Cuban Neurosciences Center, University of Ghent, Belgium and those from the Max Planck Institute for Psychiatry as well as other local and overseas institutes of higher education.

Research and It's focus

Major research outputs in neurosciences from USM are expected from the fundamental or applied neurosciences group within 2-4 years. At this current moment grants are being requested from the Ministry of Science, Technology and Innovation (MOSTI), non-governmental organization such as The National Cancer Council (MAKNA) and overseas funds. The USM brain research cluster groups hope to come out with major research topics in neuroinformatics, mental health and rehabilitation, neural instrumentation, brain mapping, interventional strategies in neurological diseases, teaching-learning strategies in children and adults as well as fundamental neurosciences.

The focus of neurosciences in Malaysia has to be supplementary and not repetitive. We should also remember that technological know how needs to be transferred to these young Malaysian researchers handling complex instruments and scientific methodologies. Previous research needs to be evaluated, as results already published may not be able to be duplicated in a Malaysian lab. Models of neuroscience research needs to be made relevant to those actual nervous system diseases common in Malaysia. Most models are different and do not extrapolate directly to the human nervous system. Diseases that needs focusing in the neurosciences in Malaysia range from smoking and drug addiction, learning disabilities, viral neurological infections, hemorrhagic strokes, etnopharmacology, the human brain mapping project, behavioral sciences, new imaging techniques and rehabilitation. Invertebrate transgenic models ranging from the worm Caenorhabditis elegans, fruit fly Drosophila melanogaster, zebra fish, Daniorerio to yeast like Saccharomyces cerevisiae may be used for some of these researchs.

Conclusion

Most developed countries in the West have surpassed Malaysia in the knowledge gained from neurosciences research with more than 50 journals dedicated to clinical, applied and fundamental neurosciences. We are practically 15 years behind the United States of America and the development of a full-fledged Malaysian neuroscientist takes at least 8 years regardless of where he/she is trained an overseas institution (8).

The vision 2020 of the government of Malaysia is to have 60 – 80 scientists per 10,000 labour force of whom 10 should be fundamental or applied neuroscientists. USM will hopefully be able to assist this development in a major way by an establishment of an Institute in the area of Neurosciences.

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REVIEW ARTICLE

THE USE OF SNPS IN PHARMACOGENOMICS STUDIES

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Pharmacogenomics is the study of how genetic makeup determines the response to a therapeutic intervention. It has the potential to revolutionize the practice of medicine by individualisation of treatment through the use of novel diagnostic tools . This new science should reduce the trial-and-error approach to the choice of treatment and thereby limit the exposure of patients to drugs that are not effective or are toxic for them. Single Nucleotide Polymorphisms (SNPs) holds the key in defining the risk of an individual's susceptibility to various illnesses and response to drugs. There is an ongoing process of identifying the common, biologically relevant SNPs, in particular those that are associated with the risk of disease. The identification and characterization of large numbers of these SNPs are necessary before we can begin to use them extensively as genetic tools. As SNP allele frequencies vary considerably across human ethnic groups and populations, the SNP consortium has opted to use an ethnically diverse panel to maximize the chances of SNP discovery. Currently most studies are biased deliberately towards coding regions and the data generated from them therefore are unlikely to reflect the overall distribution of SNPs throughout the genome. The SNP consortium protocol was designed to identify SNPs without any bias towards these coding regions. Most pharmacogenomic studies were carried out in heterogeneous clinical trial populations, using case-control or cohort association study designs employing either candidate gene or Linkage disequilibrium (LD) mapping approaches. Concerns about the required patient sample sizes, the extent of LD, the number of SNPs needed in a map, the cost of genotyping SNPs, and the interpretation of results are some of the challenges that surround this field. While LD mapping is appealing in that it is an unbiased approach and allows a comprehensive genome-wide survey, the challenges and limitations are significant. An alternative such as the candidate gene approach does offer several advantages over LD mapping. Ultimately, as all human genes are discovered, the need for random SNP markers diminishes and gene-based SNP approaches will predominate. The challenges will then be to demonstrate convincing links between genetic variation and drug responses and to translate that information into useful pharmacogenomic tests.

***Key words** : Single Nucleotide Polymorphism (SNP), Pharmacogenomics, Pharmacogenetics*

Introduction

Pharmacogenomics is one of the most promising sciences for the pharmaceutical industry to emerge in the post-genomic era.

Pharmacogenomics, can be broadly defined, as the study of the impact of genetic variation on the efficacy and toxicity of drugs, or the study of how genetic makeup determines the response to a therapeutic intervention.[1] As the volume of high quality genetic and genomic information for predicting the response to drugs become available, better clinical trials and more targeted drug development will then follow.

Pharmacogenomics has the potential to revolutionize the practice of medicine by

individualisation of treatment through the use of novel diagnostic tools . This new science should reduce the trial-and-error approach to the choice of treatment and thereby limit the exposure of patients to drugs that are not effective or are toxic for them.

Pharmacogenetics

The term pharmacogenetics is often used interchangeably with pharmacogenomics, but is used more generally to describe the study the effect of genetic factors on drug response (1).

Most of the variations or polymorphisms describe to data occur in the drug metabolizing enzymes (DMEs) or cytochrome P450 enzymes. However, polymorphisms of drug transportation genes and genes that encode protein receptors and other effectors also lead to variations in drug response.

Pharmacogenomics vs. Pharmacogenetics

The many interactions involving cytochrome P450 illustrate the importance of pharmacogenomics (2). Likewise, we now have extensive compilations of clinically relevant polymorphisms (SNPs) that influence other drug metabolism pathways. For example, it is estimated that 2-10% of the population is homozygous for non-functional CYP2D6 mutant alleles, leading to an inability to activate opioid analgesics. This would explain why there is great variability in pain relief experienced by patients receiving the same dose of codeine.

Functional genomics

Functional genomics is the study of the relationships between particular genotypes and specific phenotypes.

Pharmacoproteomics

Pharmacoproteomics is the subtyping of patients on the basis of protein analysis. This mode of characterization is a more functional representation of patient-to-patient variation than is provided by genotyping, and includes the added effects of post-translational modification. Thus, pharmacoproteomics connects the genotype with the phenotype.

Single Nucleotide PolymrophISMS (SNP)

Every individual carries two copies of each gene. Copies of a specific gene present within a population may not have identical nucleotide sequences. These single nucleotide changes are scattered throughout the genome of all species and forms the basis for human diversity. SNP occur in humans every 300-2000 base pairs along the genome (3) In principle, they may occur at any nucleotide, and for genetic epidemiological study, those that are relatively common will be of greatest interest.

The vast majority of SNPs are functionally silent, occurring in non-coding or non-regulatory regions of the genome. However, some of the SNPs lead to altered protein structure or expression. These biologically functional SNPs are considered the essence and substrate of human diversity in both health and disease.

There is an ongoing process of identifying these common, biologically relevant SNPs, in particular those that are associated with the risk of disease. Once identified and characterized, this SNP-based 'genetic profile', may be viewed as a 'fingerprint', useful in defining the risk of an individual's susceptibility to various illnesses and response to drugs.

History

In the 1980s, single nucleotide polymorphisms (SNPs) were detected using restriction enzymes to identify the presence or absence of cutting sites and scored by observing the resulting fragment length variation (4). In the 1990s, the SNP was largely replaced by the simple tandem repeat (STR) as the marker of choice for linkage studies. STRs (di-, tri- or tetranucleotide repeats) show high levels of allelic variation in the number of repeat units, are widely and evenly distributed across the human genome and can be typed using Polymerase Chain Reaction (PCR) amplification. The combination of a highly polymorphic marker set and rapid typing technology led to the development of high-throughput semi-automated systems for STR genotyping during the 1990s (5).

The late 1990s saw a reversal from the use of STRs back to SNPs, which regained favour amongst molecular geneticists. Following the completion of the Human Genome Project in 2001, there has been further increase on the number of studies as well as interests on SNPs. The main driving force behind the switch back to SNPs was a change in the type of genetic studies undertaken by the various research groups. STRs are ideal for linkage studies involving pedigree analysis to identify single genes responsible for monogenic disorders. However, more recently the need to study diseases with more complex inheritance pathways, but with a higher prevalence and hence higher social burden, such as osteoporosis,

diabetes, cardiovascular and inflammatory diseases, psychiatric disorders and most cancers, has led to a refocus on SNPs. Moreover, there has also been increasing interest in the genetics of drug response (pharmacogenetics), an understanding of which may allow the 'tailoring' of therapies on an individual basis.

The broadly familial nature of complex diseases clearly indicates a significant genetic component. However, in contrast to monogenic conditions, this genetic element is comprised of multiple gene variants each contributing a small effect. This genetic complexity may also be compounded by heterogeneity, with different combinations of gene variants giving rise to a similar phenotype. The extent of this problem is likely to be so great that the frequency of any polymorphism contributing to a disease phenotype may be only slightly elevated in a disease group when compared with unaffected controls. Unfortunately, linkage analysis has limited power to detect such small effects, and attempts to identify genes involved in complex disease using linkage-based approaches have generally proved disappointing. Association studies with large sample sizes, and involving comparisons of cases of disease with matched controls from the same population, are likely to give a greater chance of detecting small effects.

Until now, population-based case–control studies have been limited to attempts to associate one or a few 'candidate genes' with disease. This restricted approach has been due largely to the lack of appropriate genetic markers and the inadequacy of the available genotyping tools for the high-throughput approaches required for large-scale genome-wide experiments. Ironically, the STR markers that have been so successful in the study of monogenic disease will probably be of limited value in population-based studies. The high level of variation reflects high mutation rates, which are likely to confound population-based approaches (6). Furthermore, due to the large number of markers required, STR loci may be too sparse for association-based approaches.

In contrast, SNPs are abundant and are more stable than STRs due to lower mutation rates. Moreover, STR loci suffer from being 'surrogate' markers in the sense that polymorphism in the STR is used to locate an adjacent functional variant that contributes to the disease state. Variation at the STR itself rarely contributes to the phenotype. While SNPs may also act as surrogate markers, many SNPs have functional consequences if they occur in the coding or regulatory regions of a gene. Therefore, by using SNP markers, it is often possible to test for association between a phenotype and a functional variant directly. For these reasons, SNPs are preferred for drawing the high-density genetic marker maps required for one of the major thrusts in human genetics research: the unraveling of complex genetic traits.

Methods for Identification of SNPs

The identification and characterization of large numbers of SNPs are necessary before we can begin to use them extensively as genetic tools. A pool of several hundred thousand SNPs will be required as a resource for the construction of optimized marker sets for association studies.

There are five commonly used methods for SNP (or mutation) detection (7 - 11).

(1) Single strand conformation polymorphisms (SSCPs)

(2) Heteroduplex analysis

(3) Direct DNA sequencing

(4) Variant detector arrays (VDAs).

(5) DNA microarray technology.

(i) SSCP detection

For SSCP detection, the DNA fragment spanning the putative SNP is PCR amplified, denatured and run on a non-denaturing polyacrylamide gel. During the gel run, the single-stranded fragments adopt secondary structures according to their nucleotide sequences. Fragments bearing SNPs are identified as a result of their aberrant migration patterns and confirmed by sequencing.

Although SSCP is a widely used and relatively simple technique, its success rate for SNP detection, has been variable, typically ranging from 70 to 95%[7]. It is a labour-intensive method and has a relatively low throughput, although higher capacity methods using capillary- rather than gel-based detection are under development (9).

(ii) Heteroduplex analysis

Heteroduplex analysis relies on the detection of a heteroduplex formed during reannealing of the denatured strands of a PCR product derived from an individual heterozygous for the SNP. The heteroduplex can be detected as a band shift on a

gel, or by differential retention on a high-performance liquid chromatography (HPLC) column.

HPLC has rapidly become a popular method for heteroduplex-based SNP detection due to its simplicity, low cost and high rate of detection (95-100%) (12) Reasonable throughput at 10 min per sample can be achieved with commercially-available systems such as the Transgenomic Wave (13).

(iii) Direct DNA sequencing

Currently, the favoured high-throughput method for SNP detection is direct DNA sequencing. Once the sequencing reactions have been completed, a single capillary system (e.g Applied Biosystems 3700) can generate sequences from more than 1500 DNA fragments of 500 bp in 48 h with minimal human intervention. Dye-terminator sequencing chemistry will detect 95% of heterozygotes and the more expensive and labour-intensive dye-primer chemistry may identify all sequences (8).

The recently formed SNP consortium (TSC), a non-profit foundation sponsored by 10 major pharmaceutical companies and the UK Wellcome Trust, has used dye-terminator sequencing to identify and has succeeded in mapping more than 100 000 SNPs by 2001.

SNPs may also be detected *in silico* at the DNA sequence level. The wealth of sequence data deposited in public databases in recent years, in particular expressed sequence tag (EST) sequences, allows SNPs to be detected by comparing multiple versions of the same sequence from different sources.

(iv) VDA technology

VDA technology is a relatively recent addition to the high-throughput tools available for SNP detection.

This technique allows the identification of SNPs by hybridization of a PCR product to oligonucleotides arrayed on a glass chip and measuring the difference in hybridization strength between matched and mismatched oligonucleotides.

The VDA detection rate is comparable to that of dye-terminator sequencing and allows rapid scanning of large amounts of DNA sequences. For example, Wang et al[7] used this technique to identify 2500 SNPs in 2 Mb of human DNA and, more recently, Halushka et al (13) have used the same method to identify 874 SNPs in 75 candidate genes for hypertension.

Pyrosequencing

Pyrosequencing, described by Ahmadian et al (14) is a sequencing-by-synthesis method in which a cascade of enzymatic reactions yields detectable light radiation, characteristic of the incorporated nucleotides. One feature of typing SNPs with pyrosequencing is that each allelic variant, being unique in sequence, can easily be distinguished by pattern-recognition software. The software displays the allelic alternatives and allows for direct comparison with the pyrosequencing raw data.

For optimal determination of SNPs, various protocols for the order of dispensing of the nucleotides should be used. Ahmadian et al demonstrated that suitability of the technique for large-scale screening and typing of SNPs by pyrosequencing 96 samples in approximately 5 min using an automated system for parallel analysis (14).

(v) DNA Microarray Technology

The DNA microarray technology is the latest, cutting edge technology for the studies on SNPs (10, 11). It offers a biotechnological revolution with the help of DNA chemistry, silicon chip technology and optics to be used to monitor gene expression for thousands of genes in one single experiment. Briefly, 20,000 to 100,000 unique DNA molecules get applied by a robot to the surface of silicon wafers (approximately the size of a microscope slide). Using a single microarray experiment, the expression level of 20,000 to 100,000 genes could be examined in one single experiment. Microarray tools are now used on regular basis for monitoring gene expression of large number of genes and also frequently applied to DNA sequence analysis, genotyping, and molecular diagnosing. These tools can be used to distinguish and differentiate between different DNA fragments that differ by as little as a single nucleotide polymorphism (SNP), making it a powerful tool for identifying novel molecular drug targets and for elucidating mechanisms of drug action. Furthermore, microarrays can monitor the global profile of gene expression in response to specific pharmacologic agents, providing information on drug efficacy and toxicity (11).

Sample population

In addition to choosing a method for SNP detection, the population in which the SNPs are to be detected must be defined. SNP allele frequencies vary considerably across human ethnic groups and populations. The SNP consortium has opted to use

an ethnically diverse panel to maximize the chances of SNP discovery.

In their study of hyertension, Halushka et al. (15) analyzed African and Northern European populations due to known differences in prevalence and disease phenotype in these two ethnic groups. Other studies use populations with a target disease for SNP discovery, on the logic that variants contributing to the disease state should occur with higher frequencies in such cohorts (16).

Given that any polymorphism is likely to make only a small contribution to a disease phenotype and that it will be found at only a slightly higher frequency in the disease cohort compared with a control group studies with a matched non-diseased population is necessary.

Different SNP panels will be required for different studies. However, a diverse approach is necessary for the generation of a large pool of SNPs from which to draw the most appropriate panel for any given study.

Recent advances

By 1999, nearly 300 genes have undergone detailed analysis for SNP content (15, 17). Although the methods and populations used in each study were different, several useful inferences can be made. Changes in non-coding sequence and synonymous changes in coding sequence are generally more common than non-synonymous changes. This reflects greater selective pressure for reducing diversity at positions dictating amino acid identity. Transitional changes are more common than transversions, with CpG dinucleotides showing the highest mutation rate, presumably due to deamination. There is enormous diversity in SNP frequency between genes, reflecting different selective pressures on each gene as well as different mutation and recombination rates across the genome. The degree of linkage disequilibrium varies widely across different genes, again reflecting different recombination and mutation rates.

SNP consortium (TSC)

The identification and study of SNPs in specific genes has provided useful confirmation of hypothesized models for gene and genome dynamics. However, as such studies are usually biased deliberately towards coding regions, the data generated from them are unlikely to reflect the overall distribution of SNPs throughout the genome.

In contrast, the protocol used by the SNP consortium protocol was designed to identify SNPs with no bias towards the coding regions, and the 100 000 TSC SNPs mapped should generally reflect sequence diversity across the human chromosomes. However, the data set will not be completely free of bias. For example, selection will occur against sequences that are unclonable using the TSC protocol.

The TSC aimed to expand the number of SNPs identified across the genome to 300 000 by the end of 2001. Data are released quarterly via both the TSC's own web page and the SNP database dbSNP, hosted by the National Center for Biological Information (NCBI; http://www.ncbi.nlm.nih.gov/SNP/index.html). By December 2002, 2,536,021 dbSNP had been identified.

These initiatives come in response to efforts in the biotechnology industry to identify and patent large numbers of SNPs. Most notable are the efforts by Celera Genomics (Rockville, MD), Genset (Paris, France), CuraGen (New Haven, CT), and Incyte Genomics (Palo Alto, CA).

The Use of SNP Maps in Pharmacogenomics

There are two approaches (18) for the use of SNP maps in pharmacogenomics: the candidate gene approach and linkage-disequilibrium mapping.

(i) Candidate gene approach

The candidate gene approach uses biological paradigms or a prior knowledge of disease pathogenesis to identify genes relevant to disease. SNPs found in these genes are tested for statistical association with disease in patients enrolled in family, case-control, or cohort studies. These "susceptibility genes" are hypothesized to directly influence an individual's likelihood of developing the disease.

This approach has already been extended to identifying candidate genes affecting drug response. For example, gene variants in a drug-metabolizing enzyme (thiopurine methyltransferase; *TPMT*) have been linked to adverse drug reactions (19). Gene variants in a drug target (5-lipoxygenase; *ALOX5*) have been associated with variation in drug response (20) and variants in a disease susceptibility gene (apolipoprotein E; *APOE*) have been correlated with response to a cholinesterase inhibitor in Alzheimer's patients (21)

(ii) Linkage disequilibrium mapping

An alternative to the candidate gene approach

is linkage disequilibrium mapping. This approach relies on linkage disequilibrium (LD) or nonrandom association between SNPs in proximity to each other.

Tens to hundreds of thousands of anonymous SNPs need to be identified and their location in the genome mapped. Although these anonymous SNPs may fall within genes and may in fact be susceptibility SNPs, most are located in the vast noncoding DNA regions between genes and play no obvious role in drug response. Through LD, associations found, the anonymous markers can be used to identify a region of the genome that may harbor a susceptibility gene without any a priori assumptions about what or where the susceptibility gene is. Additional significant efforts using positional cloning are then required to find the specific gene and the SNPs within it that confer the underlying association.

Linkage disequilibrium mapping has been employed successfully on families with multiple affected individuals to uncover genes for monogenic diseases (22). Even though this mapping technique is now being considered in the context of association studies, it has not been successful for identifying genetic predictors of either disease or drug response in unrelated individuals.

Limitationns of SNPs as A Tool in Pharmacogenomics Analyses

Studies of the genetic basis of disease can take advantage of characteristics of familial inheritance, use of homogeneous populations and relatively straightforward case-ascertainment of affected individuals. In contrast, pharmacogenomic analyses are more complicated:

(i) Drug response is a trait whose expression can only be gauged after administration of the therapeutic compound under study. Ascertainment of responders cannot be made from the non-exposed general population and use of families, is generally precluded except in the rare instance where multiple family members are given the drug.

(ii) Clinical trials are the main source of patients for pharmacogenomic studies and these are limited in size, making estimation of linkage-disequilibrium, often impossible, and usually imprecise.

Moreover, although drugs on the market may be sold worldwide, most clinical trials of new therapies are performed in Caucasian Americans or Europeans. Pharmacogenomic studies are therefore usually limited to these genetically heterogeneous clinical trial populations. Case-control or cohort association studies are usually employed to identify candidate gene or for LD mapping.

(iii) The cost of genotyping SNPs, and the interpretation of results are also problems.

Sample size:

The number of patients required to find a statistically significant association between an SNP and an abnormal drug response depends on a number of factors, including the frequency of the drug response, the proportion of patients having the SNP allele, the minimum detectable drug effect using existing diagnostic criteria, the level of statistical significance (*p* value or probability of missing a true difference), and the power (the probability of not missing a true association) required.

The SNPs most likely to have a direct impact on the protein product of a gene are coding region SNPs (cSNPs) that change amino acid sequence, and SNPs in gene regulatory regions, which control protein levels. Coding SNPs that confer association in a recessive fashion (where two copies of the cSNP are required) may occur in too few patients to be useful as pharmacogenomic markers (17, 20).

The extent of LD

(Estimating the number of markers needed in a SNP map)

Genome-wide SNP LD mapping is predicated on the assumption that LD exists between SNPs. The extent of LD occurs as a consequence of many factors, including population admixture, genetic drift, mutation, and natural selection (23). For genetic distances measured in kilobases (kb) of DNA, LD tends to decline with larger distance between SNPs in the range of 10–100 kb. Over shorter genetic distances the degree of LD is highly variable from one genomic region to the next. In some genomic regions, LD extends over several thousands of kilobases, whereas in other genomic regions surrounding single genes, LD can be quite small. Theoretical estimates of the average extent of LD in the human genome vary widely, ranging from <100 kb to <3 kb (24, 25).

As increasing amounts of genetic data become available, the true extent of LD throughout the genome can be tested empirically.

Understanding the average extent of LD is useful for estimating the number of markers needed in a SNP map and the strength of the association that the markers are capable of detecting.

Linkage disequilibrium mapping requires that a susceptibility allele be detectable with a marker that lies within the interval afforded by the SNP map density. Given the estimated 3 billion bp size of the human genome, a minimum of 30,000 to 500,000 evenly spaced SNP markers would be needed to have a marker every 100 to 6 kb (i.e., within the range of LD). Maps currently under construction range in size from 60,000 to 300,000 SNPs (26) resulting in a SNP mapped on average every 50 to 10 kb, respectively. Whereas this density may be useful for uncovering SNPs in genomic regions with extensive LD, genes in regions where LD is less extensive may be missed. To improve the chance of successfully identifying susceptibility SNPs through LD, high-density maps will be required.

The strength of LD

The strength of LD will also affect the magnitude of an association. A marker in LD with a susceptibility SNP will yield a relative risk that is smaller than if the susceptibility SNP were tested directly. D' is a measure of linkage disequilibrium (27) that ranges in value from 0 (no disequillibrium) to 1.0 (complete disequilibrium). The weaker the LD between marker and susceptibility SNPs, the smaller the relative risk, and the more difficult the association is to detect unless the sample size is increased proportionately. This situation becomes even more complex when large differences in allele frequencies exist between markers and susceptibility SNPs. If marker allele frequencies are substantially different from the susceptibility allele frequency, then the required sample size, the number of markers, or both will need to be dramatically increased.

Sample sizes for LD mapping

As LD mapping requires testing of hundreds of thousands of markers, the chance of producing false positive results is high. To reduce the number of false positive results, a correction can be applied whereby a more stringent cutoff is used for establishing statistical significance. To achieve an overall 5% false positive rate when 100,000 independent markers are tested, a *p* value of 0.0000005 should be used (18)

Cost-of-genotyping

One of the major challenges of LD mapping is the need to genotype each person in the study for every one of the 60,000–500,000 SNPs in the map. Several genotyping platforms are available today, including nucleic acid hybridization on filters or chips, single-strand conformational polymorphism (SSCP), and primer extension-based methods. Although these genotyping technologies are robust, at a current average price of one dollar per genotype, their use in large-scale SNP genotyping studies may be very expensive. Even at one cent per genotype, the cost per person in a typical association study testing 100,000 SNPs will be about $1,000, possibly adding $1 million to the cost of a clinical trial. A method that involves pooling of patient DNA samples has been suggested to reduce the overall number of genotypes needed (28). However, pooling presents technical challenges and drawbacks because it prohibits subgroup and haplotype analysis. Significant advances are required to make extensive genotyping a standard part of clinical trials.

Interpretation of results

Interpretation of data from pharmacogenomic association studies is challenging. Two questions should be asked: "Is any association detected real" and "are the results useful?" Studies yielding statistically significant results are often considered real even though the results are rarely replicated. Consistency of results between studies is a major issue for both candidate gene studies and studies employing genome-wide LD mapping. Furthermore, the use of low *p* values is recommended to allow for the vast number of genetic hypotheses that will be tested collectively in the field. Very low *p* values and reproducibility should be part of any set of criteria for judging the reality of associations, especially when the results trigger further investment in positional cloning (for LD mapping studies) or are the basis of diagnostic tests used to convey risks to patients or direct a course of therapy.

How strongly will SNPs be associated with drug response?

The use of a SNP associated with drug response can be judged in terms of how well it predicts drug response in patients, and the proportion of patients who will benefit from the test. In the recent paper by Drazen and coworkers (20), an *ALOX5* genotype was associated with response to an antiasthmatic compound. The genotype had a 100% positive predictive value for nonresponse to the drug. However, because the susceptibility genotype is uncommon (6–9% of patients), less than 10% of the nonresponse can be attributed to this

genotype. Therefore, if patients with the susceptibility genotype avoided taking the drug, the efficacy would only improve from 46% to 51% in the remaining patients. Whereas the test may benefit a few patients who would otherwise receive unnecessary and ineffective therapy, it will not identify the majority of nonresponders. In order to have practical utility, additional SNPs will need to be identified and used together with the *ALOX5* SNP as a battery where each individual SNP explains a small portion of drug response. There may of course also be non-genetic causes of non-response.

The"effect-size"

The "effect size" of an association in pharmacogenomics is the likelihood of response to a drug in individuals with the susceptibility allele compared with those without the allele. Depending on whether the study design is a cohort or case-control, the magnitude of the effect is usually expressed as a relative risk (RR) or as an odd ratio (OR), respectively. Deviations from the baseline value of 1.0 for either measure indicate increased or decreased likelihood of response. Relative risk estimates for common diseases are expected to be low, in the range of 1.5 to 3.0, owing to their multifactorial nature.

Drug response is just as complex as disease genetics, resulting not only from underlying genotypic variation at many loci, but also from variation at the level of gene expression, post-translational modification of proteins, drug dose, drug interactions, diet, and other nongenetic factors. Therefore, usually individual genes will be associated with relatively small effects on drug response. In fact, pharmacogenomic markers reported to date confer only about a twofold increased likelihood of response (e.g. RR = 2.0) (18)

Future-prospects

While LD mapping is appealing in that it is an unbiased approach and allows a comprehensive genome-wide survey, limitations are significant. A number of challenges need to be overcome before the value of a high-density SNP map can be maximised for practical use. Concerns about the high price of genotyping are being addressed but it may be several years before the price of genotyping large populations becomes acceptable. In addition, availability of large patient populations will be crucial for discovering and validating SNPs. The extent of LD and success in detecting associations with small effect that will identify situations for which SNP LD mapping could work.

The candidate gene approach is an appealing alternative to the LD mapping. It is a proven method. Genotyping a limited number of candidate SNPs is already economically feasible with this method and no assumptions are made about LD. The required sample sizes are consistent with the number of subjects recruited in current clinical trials.

As more human genes are discovered, the need for random SNP markers will diminish and gene-based SNP approaches will predominate. The real challenges will then be to demonstrate convincing links between genetic variation and drug responses and to translate that information into useful pharmacogenomic tests.

How likely will individualized pharmacotherapy, within a pharmacogenetic framework, become a reality? While the technical basis for these developments is in place today and appears quite logical, two tasks are needed:

First, the creation of the necessary knowledge base for genetic risk profiling, an enormous and dauntingly difficult task and second, the acceptance of these new approaches by the general public. The experience with patient advocacy groups for single gene disorders shows that efforts to find any causative gene usually find strong support, being recognized as the first, essential step towards treatment, cure or prevention.

Indeed patients may well become the driving force behind the development of this vision of integrated and individualized medicine as ultimately, it is they who stand to gain most. However, as corporations stand to gain much too, vigilance is required to ensure that societal agenda of maximum health gain is not hijacked to be transformed into one aimed at maximizing profits. Some would argue that this is the major challenge to genomic medicine.

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ORIGINAL ARTICLE

EVALUATION OF THE TERATOGENICITY OF AQUEOUS EXTRACT OF *Labisia pumila var. alata* IN RATS

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A dose range study to assess the teratogenic potential of aqueous extract of *Labisia pumila var. alata* (Kacip Fatimah) was conducted in rodents. The extract at doses of 0 (control), 2, 20, 200, 400, 1000 mg/kg/day were respectively administered by gavaging to 6 groups of pregnant Sprague Dawley rats from day 6 through day 16 of pregnancy and sacrificed on day 21. No significant agent-related effects including changes in maternal body weight (MBW) nor weight gain were observed. The corrected maternal body weights (CMBW) were slightly higher in animals receiving low dose extracts (2 mg/kg/day) as compared to all groups of animals. However, body weight differences were not statistically significant. Gravid uterine weight, number of corpora lutea, number of implantation sites, percentage of foetal resorptions, number of life foetuses, foetal weight and foetal sex ratio showed no significant differences among all group animals. None of the foetuses from all dams showed evidence of external congenital malformations. These findings may suggest that aqueous extracts of *Labisia pumila var. alata* up to 1000 mg/kg/day statistically do not show any significant teratogenic effects in rats but do affect the maternal body weight and this is dose dependent.

Key words *:Labisia pumila var. alata, aqueous extract, teratogenicity*

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Introduction

Labisia pumila or popularly known in Malaysia as Kacip Fatimah (KF) is a member of the small genus of slightly woody plants of the family Myrsinaceae (1). There are at least four known varieties of *Labisia pumila* found in Malaysia. Though, only three of them are popularly used by the Malays and are recognized as *Labisia pumila var. pumila* (LPP), *Labisia pumila var. alata* (LPA) and *Labisia pumila var. lanceolata* (LPL) (2).

This plant has been utilized by many generations of Malay women for the purpose of inducing and in the facilitation of labour. Water decoctions of the root or whole plant were and are still given to pregnant woman one to two months prior to childbirth (1, 2). It is also used as a postpartum medication (2) in the form of mixed preparation to help in the contraction of uterus. Further, KF is reported to delay fertility and to help regain body strength of mothers (3).

KF is very popular amongst the local womenfolk and it is frequently consumed by women of reproductive age. As a result of this popularity, to date several commercial products in the form of capsules (4) and canned drinks containing this herb have emerged in the Malaysian market (5). These products are claimed to contain the grinded or extracted parts of the plant (4, 5). However, there is no standardized preparation or any scientific data authenticating or providing evaluation of the quality, safety and efficacy of this herb (4). Furthermore, little is known about the chemical constituents of this plant. No international publication regarding to this plant available as well (6).

Since KF is consumed by women during reproductive period, it is reasonable that the present study focus on accessing the possible teratogenic

*Table 1: Reproductive and foetal parameters in rats treated with aqueous extract of **Labisia pumila var. alata** (LPE) from day 6 through day 16 of pregnancy.*

Parameters	LPE (mg/kg/day)					
	0 (Control)	2	20	200	400	1000
No. of pregnant rats	8	8	8	8	8	8
Percentage of pregnancy (%)	100	100	100	100	100	100
Change in body weight compared to expected body weight (%)	0	+ 5.19	+ 1.87	+ 3.98	- 3.97	-0.55
Gravid uterine weight (g) [a]	63.22 (1.68)	57.71 (7.18)	50.31 (9.29)	51.57 (6.55)	59.48 (5.40)	65.03 (2.93)
No. of corpora lutea/litter [a]	10.13 (0.67)	11.13 (0.52)	10.25 (0.80)	10.88 (0.72)	10.38 (0.65)	11.75 (0.31)
No. of implantation sites/litter [a]	10.00 (0.60)	9.50 (1.15)	8.13 (1.47)	8.88 (1.11)	9.38 (0.82)	11.13 (0.40)
% of pre-implantation loss/litter [b]	0	8.55 (34.55)	3.57 (57.50)	15.55 (33.22)	3.57 (24.31)	0 (15.72)
% of post implantation death (resorption)/litter [b]	0	0	0	0 (24.98)	0	0 (12.50)
Litters with early resorption	0	1	1	2	1	2
Litters with late resorption	0	0	0	0	0	0
No. of life foetuses/litter [a]	10.00(0.60)	8.88 (1.09)	8.00 (1.55)	8.38 (1.35)	9.25 (0.82)	10.63 (0.46)
Pups male: female ratio	1.50:1	1.03:1	1:1	1:1.16	1:1.47	1:1.02

[a] Mean (SEM).

[b] Median (IQR)

* Significantly different from the control, $P < 0.05$

and reproductive toxicity of this herb. A teratogenic agent could be defined as any substance which increases the chances of offspring being born with a gross structural or functionally abnormality (7, 8). The purposes of conducting this study are to ensure and establish the safety profiles of KF by evaluating the effects of the aqueous extract of *Labisia pumila var. alata* (LPE) on embryonic development (teratogenic study) in rats through Segment II Study.

The study was started by looking at possible dose related teratogenic properties of the herb (Segment II– Dose range study). If it is proven that the extract has potential teratogenic properties, then a definitive teratogenic study will be conducted (9, 10). The results obtained are expected to provide useful information on the safety profile of this herb and thereby help to alleviate concerns over its use.

Materials and Methodology

Research and ethical committee approval

Proposal of the Evaluation of the Teratogenicity (Segment II) was submitted to research and ethics committee for evaluation and was approved by Universiti Sains Malaysia's Animal Ethics Committee (USM/PPSF/050(1) Jld.1) and Universiti Sains Malaysia Health Campus's Animals Research and Ethics Committee (No 013).

Plant materials

The standardized aqueous extract of *Labisia pumila var. alata* (LPE) was provided by Institute for Medical Research (IMR), Malaysia. The raw material was previously identified and authenticated by ethno botanist of the Forest Research Institute of Malaysia (FRIM). Subsequently, the plant was sent to Chemical Engineering Pilot Plant, Universiti Teknologi Malaysia for further processing. The dosage preparation was carried out at the Pharmacology Laboratory, Universiti Sains Malaysia Health Campus using the lyophilized powdered sample obtained from the IMR.

Dosage preparation

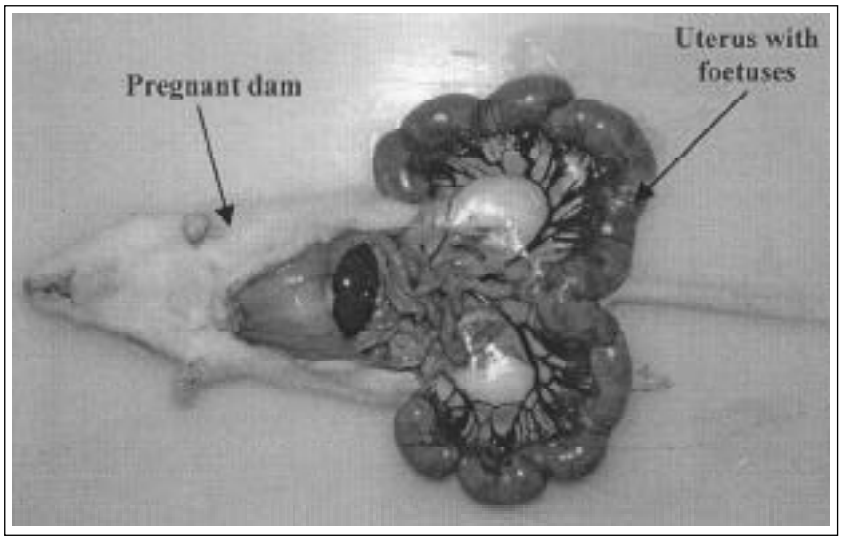
The Kacip Fatimah (KF) or *Labisia pumila* dried extract (LPE) was reconstituted with distilled water at 5 different dosages. Doses selected in this experiment were based on reported pharmacological dose (dose gives maximum favorable effect without adverse effect) which is 16.7 mg/kg/day (11).

The prepared extract were kept in small aliquots 0.2 or 0.6ml at (-40^{0}C) for experiments at the following concentrations: Group 1, Control group, LPE 0 mg/kg/day; Group 2, LPE 2 mg/kg/day; Group 3, LPE 20 mg/kg/day; Group 4, LPE 200 mg/kg/day; Group 5, LPE 400 mg/kg/day and Group 6, LPE 1000 mg/kg/day.

Animals

Female, virgin, Sprague Dawley rats weighing between 180 – 200 g and proven fertile adult males of body weights (b.w) between 200 – 250 g for mating purposes were used in this experiment. These animals were procured by Animal

Figure 1: Picture showing the pregnant dam of control group animal at sacrificed. Treatment was given from D6 through D16 of pregnancy. Dam was sacrificed on D21 of pregnancy. There were no abnormal gross findings at autopsy in all animals throughout study.



House, Universiti Sains Malaysia Health Campus. All rats were healthy and maintained according to the USM ethical guidelines under standard laboratory conditions. They were acclimatized to the laboratory environment for 2 weeks prior to use. Animals were housed at 20 ± 2^0C with 12 h light / dark cycle (lights on from 0700 to 1900). The animals had free access to commercially obtained pelleted rat chow and water *ad libitum*.

Mating procedures

Daily vaginal smear was performed in all females. Pregnancy was then induced by separately caging each female with a confirmed fertile male in the evening of proestrus. A sperm positive vaginal smear in the next morning was considered as day 0 (D0) of pregnancy (pc) (9, 10, 12).

Treatment

Forty-eight (48) pregnant rats were randomly divided into 6 groups (8 animals per group). Group 1 (Control group) received vehicle treatment (distilled water (DW)) 0.2ml daily, whereas Group 2, 3, 4, 5 and 6 received herbal extract at a dose of 2, 20, 200, 400 (0.2ml) and 1000 mg/kg/day (0.6ml) respectively. All treatment were administered during D6 through D16pc (period of organogenesis) which is defined as the critical period for the structural development span of the embryonic stage for rats (13, 14, 15).

Behavioral observations

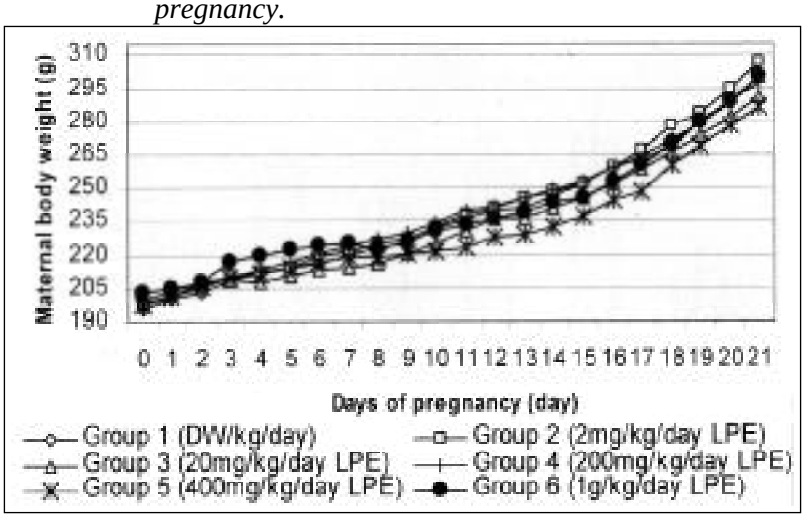
During the treatment and post-treatment periods, the animals were observed closely for any signs of toxicity. Daily body weight was recorded for all animals.

Assessment on dams and foetuses

On D21 of pregnancy, all dams were sacrificed by CO_2 asphyxiation and were immediately laparotomised. Examinations of maternal visceral organs such as uterus, ovaries, adrenal glands, liver, spleen, kidney and gut were then performed macroscopically. The gravid uterus and ovaries were subsequently removed, cleared of adhering tissue and weighed. The isolated uteri were cut open and position of implantations, early resorption (no embryonic tissue visible at termination) or late resorption (placental and embryonic tissue visible at termination) and dead or live foetuses were counted. Uteri of non-pregnant dams were stained in 0.5% ammonium sulfide to confirm the absence of implantation sites (9).

The umbilical cord of each foetus was cut and foetuses were removed, blotted dry, weighed and checked for their sex. Male and female foetuses were differentiated by examination of the location of the genital papilla (which is further away from the base of the tail in males) (16). Head, eyes, palate, nares, limbs, neck, spine, chest, abdomen, orifices, tails and genitals were examined under dissecting stereomicroscope (Motic Digital Stereomicroscope, DM-143 PAL System).

Figure 2: Graph showing the maternal body weights of treated and control group animals. Treatment was given from D6 through D16 of pregnancy. There were no significant differences in the maternal body weights of treated and control group animals from day 0 through day 21 of pregnancy.



Outcome measures

Several parameters were recorded in this study which includes fates of females such as survivability and death. Clinical signs, maternal visceral changes, daily maternal body weight (MBW) (D0-21) and their weight gain (D6-16, D6-21 and D16-21) were monitored and recorded.

Gravid uterine weight, corrected maternal body weight (maternal body weight D21 – gravid uterus weight) (CMBW), percentage change in body weight compared to expected body weight (corrected maternal body weight of control animal-corrected maternal body weight of treated animal / corrected maternal body weight of control animal × 100), number of corpora lutea per litter, number of implantation sites per litter, percentage of pre implantation loss (no. corpora lutea – no. implantation / no. corpora lutea × 100), number of resorption sites (early and late resorption) and percentage of post implantation death (no. implantation – no. live foetuses / no. implantation × 100) were noted.

The live foetuses were counted and weighed, sex distributed (male and female) and organs checked for external abnormality. Any malformation was reported as per treatment group and per litter.

Statistical evaluation

Firstly, all the parameters were checked for normality using Normality Test as well as Levene's Test to check for homogeneity of variance and to determine if the groups have unequal variances at the 5% level of significance (17). Subsequently, to the Normality and Levene's Test (if the data showed normal distribution and homogeny), parametric test was performed. Nonparametric test was used for skewed and non homogenous data. The 0.05 level of probability was used as the criterion for significance.

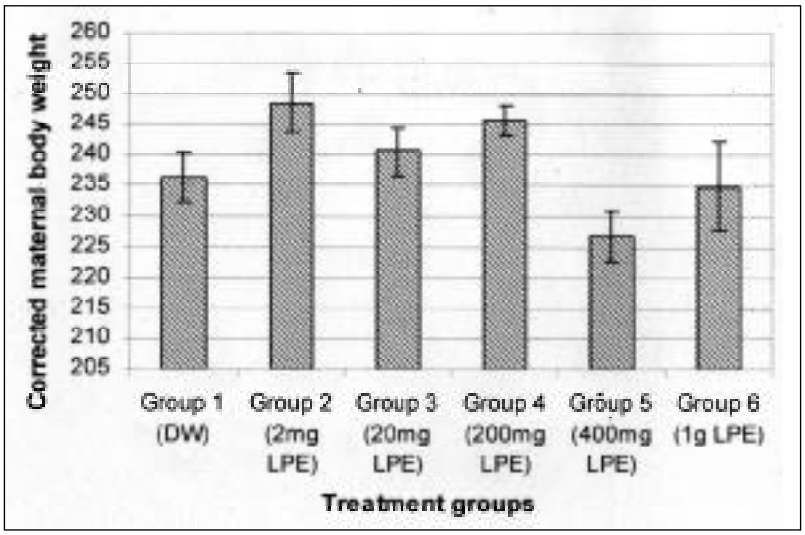
Data on maternal body weight (MBW) (D0-21 pc) was analyzed by General Linear Model Repeated Measures. Maternal weight gain, corrected maternal body weight (CMBW), maternal organ weights (ovaries and uterus), number of corpora lutea per litter, number of implantation sites per litter, number of life foetuses and foetal body weight were analyzed by One-way analysis of variance (ANOVA), followed by Scheffe Test if differences were found. Additionally, Kruskal Wallis test (nonparametric) was used to assess the overall effects of percentage of pre-implantation loss and percentage of post-implantation death. The parametric data were evaluated and expressed as Mean (Standard Error of Mean) (SEM) or ratio while the non-parametric data were expressed as Median (Interquartile Range) (IQR).

Results

Fates of females and other clinical signs

Treatment with *Labisia pumila* aqueous extract (LPE) did not adversely affect the progress of pregnancy and clinical condition of the rats (Figure 1). All dams did not exhibit abnormal

Figure 3: Histogram showing the means of corrected maternal body weights of treated and control group animals. Treatment was given from D6 through D16 of pregnancy. There were no significant differences in the means of corrected maternal body weights among all group animals.



findings as they grew normally and healthily. No mortality and morbidity were recorded throughout this study. There were also no abnormal findings at autopsy.

Maternal body weight (MBW) (D0-21), weight gain (D6-16, D6-21 & D16-21), gravid uterine weight and corrected maternal body weight (CMBW)

There were no significant differences (P>0.05, GLM Repeated Measures) in maternal body weights (MBW) on D0-21 pc (Figure 2) whilst gravid uterine weight and maternal weight gain from D6-16, D6-21 & D16-21 (one-way ANOVA) (Table 1) between treated and control group animals. The corrected maternal body weights (CMBW) were slightly reduced in groups treated with 400 and 1000 mg/kg/day LPE as compared to control. While animals that received 2 mg LPE/kg/day (Group 2) showed the highest corrected maternal body weight among all group of animals. The animals that received extract of 400 mg/kg/day gained less weight. The differences in the corrected maternal body weight were however, not statistically significance (Figure 3).

Maternal visceral changes and organ weights

No gross maternal visceral changes that were considered unusual were observed both in treated and the control group animals.

There was no significant difference (P>0.05, one-way ANOVA) in maternal reproductive organ weights (ovaries, adrenal glands and uterus) among all group animals (data not shown).

Number of corpora lutea and number of implantation sites per litter

There were no significant differences (P>0.05) in number of corpora lutea and number of implantation sites per litter between treated and control group animals (Table 1).

Post-implantation death and foetal resorption

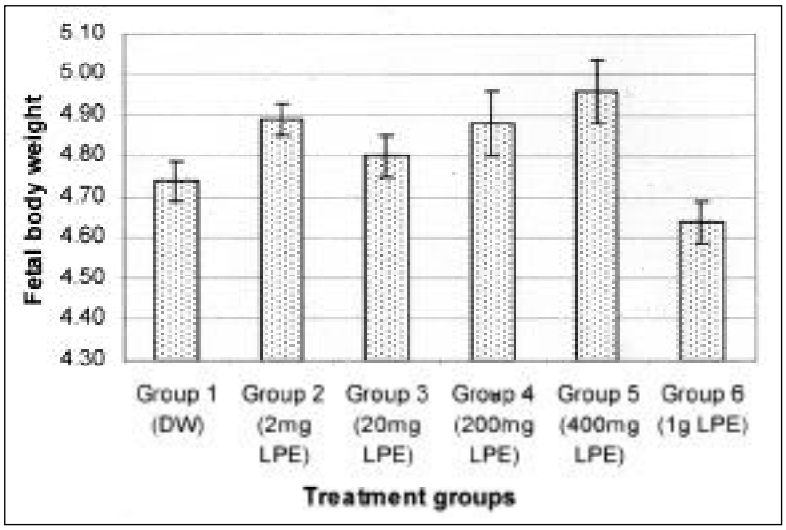
There was no statistical differences (P>0.05; Kruskal Wallis test) in percentage of post implantation death between treated and control group animals.

Few early foetal resorptions and post-implantation death were noted in the treated group of animals. Control group animal given distilled water did not show any foetal resorption. However, there were no significant differences (P>0.05) when compared to the control group for both the early resorptions and post-implantation death (Table 1). No late resorption was noted in any of the animals.

Number of life foetuses per litter

All foetuses in every group were alive at autopsy (D21). This was confirmed by observation of breathing or when they could be induced to respond. There was a slightly reduction in the number of life foetuses in animals of Group 2, 3, 4 and 5 as compared to the control group. However,

Figure 4: Histogram showing the means of fetal body weights of treated and control group animals. Treatment was given from D6 through D16 of pregnancy. There were no significant differences in the means of fetal body weights among all group animals.



animals in Group 6 that received high dose (1000 mg/kg/day LPE) showed the highest number of life foetuses among all groups of animals (Table 1). It was however, not significantly different (P>0.05) when compared to all groups of animals.

Sex distribution and foetal weight (male and female)

Based on one-way ANOVA, the number of male and female foetuses were similar (P>0.05) within and among all group animals. The overall male: female foetus sex ratio was equal (1:1.01).

Likewise, no significant difference (P>0.05, one-way ANOVA) was observed in foetal weight for all group animals (Figure 4). Foetal body weight of animals which received herbal extracts was commonly higher than control group except for those in Group 6 (1000 mg/kg/day LPE) but no significant difference was seen when compared to control group.

External malformations

Based on the observations and examinations under dissecting stereomicroscope, there were no external malformations noted on the head, eyes, palate, nares, limbs, neck, spine, chest, abdomen, orifices, tails and genitals of all pups of treated and control group animals (Figure 5). Overall, a total of 361 pups of treated group animals and 80 pups of control group did not show evidence of external congenital malformations.

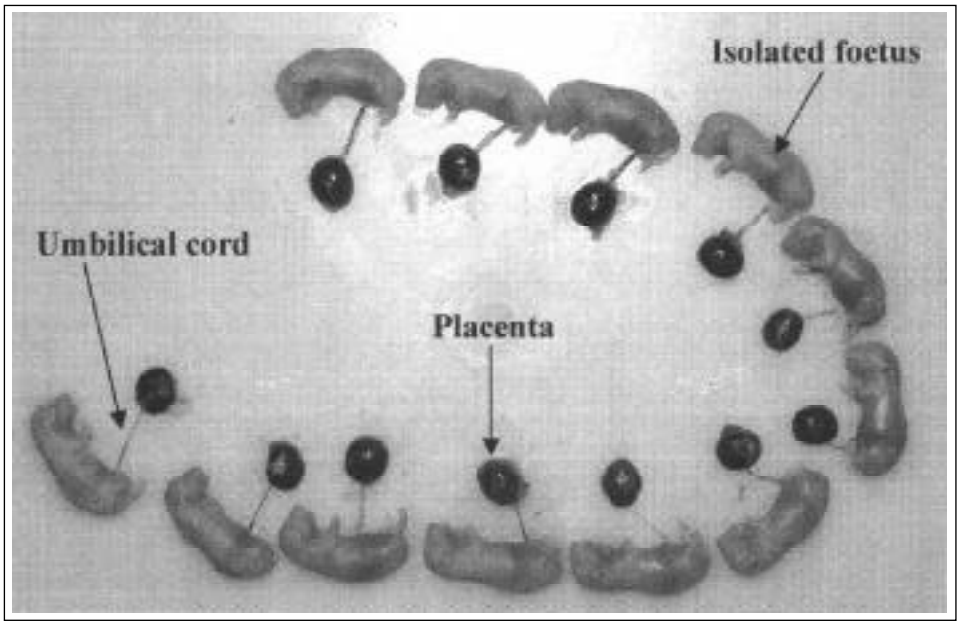
Discussion

The literature provides limited number of controlled studies with Kacip Fatimah. The highly popular nature of Kacip Fatimah amongst Malay women warrants a detailed scientific study. In this regard, the present study was carried out using LPE at doses of 2 to 1000 mg/kg/day. This dose range is selected to reflect the doses used in the practice of the Malay women. This study is the first to evaluate the teratogenicity of this herb.

Results obtained showed that the exposure of rats to the LPE of up to 1000 mg/kg daily (as suggested by most guidelines for toxicity studies) during period of organogenesis did not show any significant deleterious effect on rats. Slightly less maternal weight gain was noted in animals that received extract (400 mg/kg) during period of organogenesis (D6-16 of pregnancy). However, no significant difference was found when compared to the control group. This decrease was inconsistent and may be attributed to some possible toxicological effect but may not be toxicologically significance. Additionally, there were no statistical differences (P> 0.05) in maternal body weights (MBW) of treated and control group animals from D0 through D21 of pregnancy. These findings suggest that the herb do not have any significant deleterious effect on the progression of pregnancy in rats.

The corrected maternal body weight (CMBW) was slightly higher in animals receiving low dose (2 mg/kg) compared to the control and those receiving higher doses of the herbal extract

Figure 5: Picture showing the fetuses of control group dam. Treatment was given from D6 through D16 of pregnancy. Caesarean section was performed on D21 of pregnancy. Examination under dissecting stereo microscope did not indicate any external malformation in any of the fetuses of all groups of animals.



(20, 200, 400 and 1000 mg/kg) in the present study. However, they were not statistically different when compared to all groups of animals. The percentage change in body weight also showed a similar trend to finding of the corrected maternal body weight. These suggest that the herb may affect maternal body weight and that it was dose dependent.

Percentages of early foetal resorption and post implantation death showed no significant findings (P>0.05). This result is encouraging as it may imply that LPE is unlikely to be fetotoxic in rats. Incidence of early or late resorptions in the control animals were absence in the present study and this could be considered normal. The small number of animals used in this study could well be the reason for the reproductive effects (early foetal resorption and post implantation death) not showing up in the control females. Foetal resorptions on the other hand can still occur in untreated as well as treated rodents (18).

Gravid uterine weights were lower in treated group of animals compared to control group except for those receiving the highest dose (1000 mg/kg/day). However, the differences were not statistically significant. In addition, uterine weight can be influenced by litter size, viable foetuses and foetal gender where females tend to be smaller than males (16, 18).

Foetal body weight, on the other hand was higher in the treated group animals (Group 2, 3, 4 and 5) except for Group 6 but show no statistically significant difference when compared to control group. The slightly higher foetal body weight could be due to compensatory effect of slightly lower number of life foetuses in the specific treated groups (Group 2, 3, 4 and 5) (refer to Table 1). Foetal body weights are influenced by intrauterine growth rates, litter size and gestation length. Individual foetuses in large litters tend to be smaller than foetuses in smaller litters. Thus, reduction in their weights that can be attributed to the large litter size should not be considered as an adverse effect unless the increased litter size is treatment related (18). Examination under dissecting stereomicroscope did not indicate any gross malformation in any of the foetuses. The male and female foetal ratio was equal in control and treated group animals.

The above findings may show some evidence that *Labisia pumila* aqueous extract lacks any observable fetotoxic effect when given at doses of up to 1000 mg/kg/day to rats during the period of organogenesis. Further, no external malformation in the pups observed in this study substantiates the above conclusion.

Conclusion

In conclusion, the present data demonstrate that Kacip Fatimah or *Labisia pumila* aqueous extract might affect the body weight of dams and the effect was dose dependent. The differences in

maternal weight gained in the treated group of animals did not adversely affect foetal growth and well being as indicated by a reasonably good foetal body weight. Early foetal resorption (early post implantation death) was not significantly increased in treated group animals. No late foetal resorption was noted in any of the rats. None of foetuses in treated group animals showed evidence of external congenital malformations. Findings of the current study suggest that aqueous extract of *Labisia pumila* did not cause significant fetotoxic effect in rats. No observable adverse effect level (NOAEL) for LPE in this study was determined at 1000 mg/kg/day. However, this study alone is insufficient to make an overall conclusion of teratogenicity. Thus, to clarify this observation, a more definitive study and a thorough investigation using a much larger cohort of animals are required to confirm the safety profile of this herb on the female. In addition, human studies utilizing cell-lines should be designed to measure the developmental toxicity and not just teratogenicity prior to comparative assessment between species.

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Malaysian Journal of Medical Sciences, Vol. 12, No. 2, July 2005 (22-26)

ORIGINAL ARTICLE

IN VITRO EFFECTS OF *PLANTAGO MAJOR* EXTRACT ON UROLITHIASIS

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The study was carried out to determine the *in vitro* effect of *Plantago major* extract on calcium oxalate crystals and to compare the effects of *Plantago major* extract with clinically used drugs like allopurinol and potassium citrate (positive controls). Modified Schneider slide gel method was used for the *in vitro* study and the crystals formed were measured by Image Analyser system KS 300, 3.0 Carl Zeiss. The concentrations of *Plantago major* extract used were from 100ppm to 350ppm. *Plantago major* extract at concentrations in the range of (100ppm-350ppm) significantly inhibited the size of calcium oxate crystals (dihydrate variety) against negative control ($p<0.05$) and against positive controls($p<0.05$). However the inhibition concentration 50 (IC_{50}) values on the size of calcium oxalate crystal for the extract, potassium citrate and allopurinol were 300ppm, 350ppm and 450ppm respectively. Extract of *Plantago major* also has inhibition effect on the number of crystals but it was not significant. In conclusion extract of *Plantago major* was better than allopurinol and potassium citrate in inhibiting the size of the calcium oxalate crystal *in-vitro*.

Key words *: Plantago Major, urolithiasis, in Vitro*

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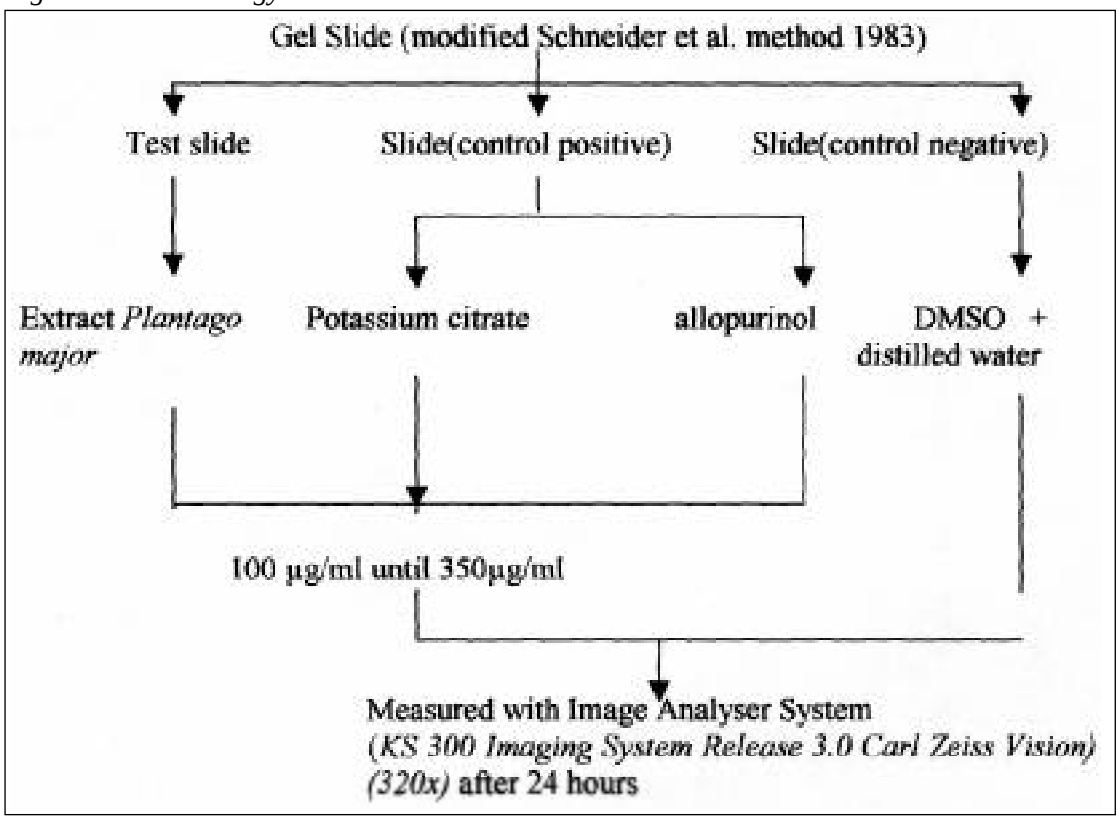
Introduction

Urolithiasis is a condition where there is a formation of stone in the urinary system, i.e in the kidney, ureter, urinary bladder or in the urethra (1). Generally there are five different types of stones of which calcium oxalate is the most common stone (80%), calcium phosphate stone (5%), magnesium ammonium phosphate, cistine and uric acid stone (2). There are two varieties in calcium oxalate stone, i.e monohydrate type (in the form of dump bell or oval) and dihydrate type (in the form of double pyramid) (3). The cause is multifactorial including diet, genetic and environmental (4). Many treatments have been tried for the treatment of urolithiasis in Malaysia and other parts of the World. It recurs back within five years (50%) and there is no one standard treatment that can prevent the recurrences (5). *Plantago Major* Linn. belonging to the family *Plantaginaceae* is a perennial herb found wild throughout the whole of Europe and temperate Asia (6). Every part of the plant has been used in many traditional medicines to treat cough, diarrhoea, dysentery, urinary tract calculus (6,7,8). Allopurinol (*Zyloric*) is a uricosuric agent that has been clinically used for the follow up patients with stone as it reduced the production of uric acid in urine and in fact uric acid may be a nidus for calcium oxalate crystals (9). Potassium citrate is a low molecular weight inhibitor for crystallization. The present study reports on the inhibiting effects of the ethanol extract of *Plantago Major* on calcium oxalate crystals *in vitro* and to compare the effects of this extract with the clinically used drugs like allopurinol and potassium citrate.

Materials and Methods

The present study was carried out in Makmal 1 of Jabatan Sains Bioperubatan, Fakulti Sains

Figure 1: Methology



Kesihatan Bersekutu in the year 2003-2004. The whole plant of *Plantago major* was collected from Cameron Highland and was identified at the Malaysian Agricultural Research & Development Institute (MARDI). The sample studied was the ethanol extract of *Plantago major* (the whole plant) soxhlet extracted after drying at room temperature for a week (10). Each extract was diluted with dimethylsulphoxide (DMSO) to get different concentrations of the *plantago major* extract, allopurinol and potassium citrate. Dimethyl sulphoxide (DMSO) was used as a negative control and allopurinol standard and potassium citrate were used as positive controls. Allopurinol standard of 0.1mg/ml was obtained by crushing Allopurinol tablets (100mg) into power and dissolving 0.001g of the powder in 5ml DMSO. The solution was then diluted further with distilled water to obtain a final concentration of 0.1mg/ml. The slides were coated with 1.5ml of 1% Bactoagar and each slide was

Figure 2 : Effects of Plantago major *extract, potassium citrate and Allopurinol on the size of calcium oxalate crystals.*

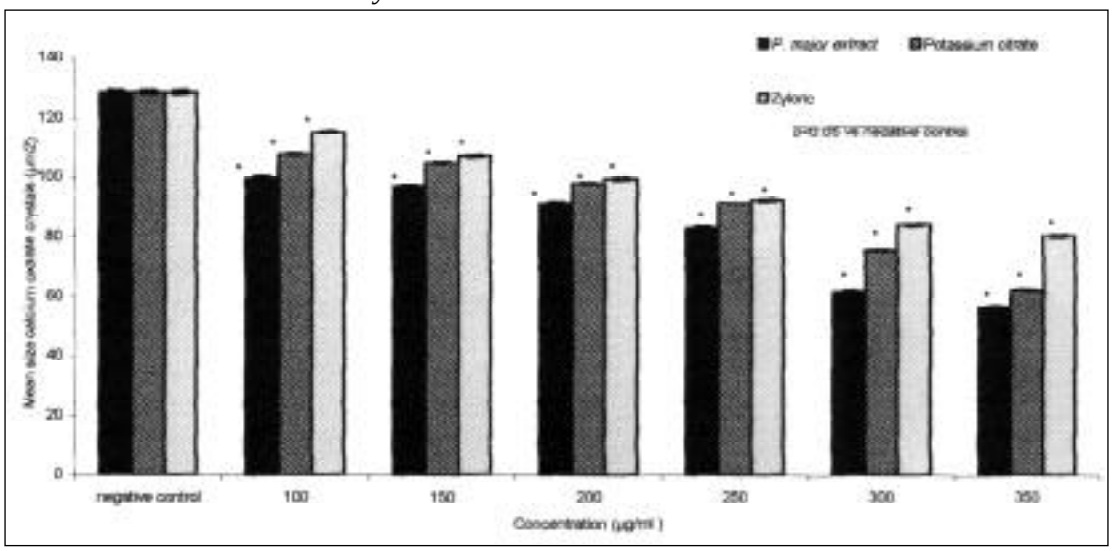
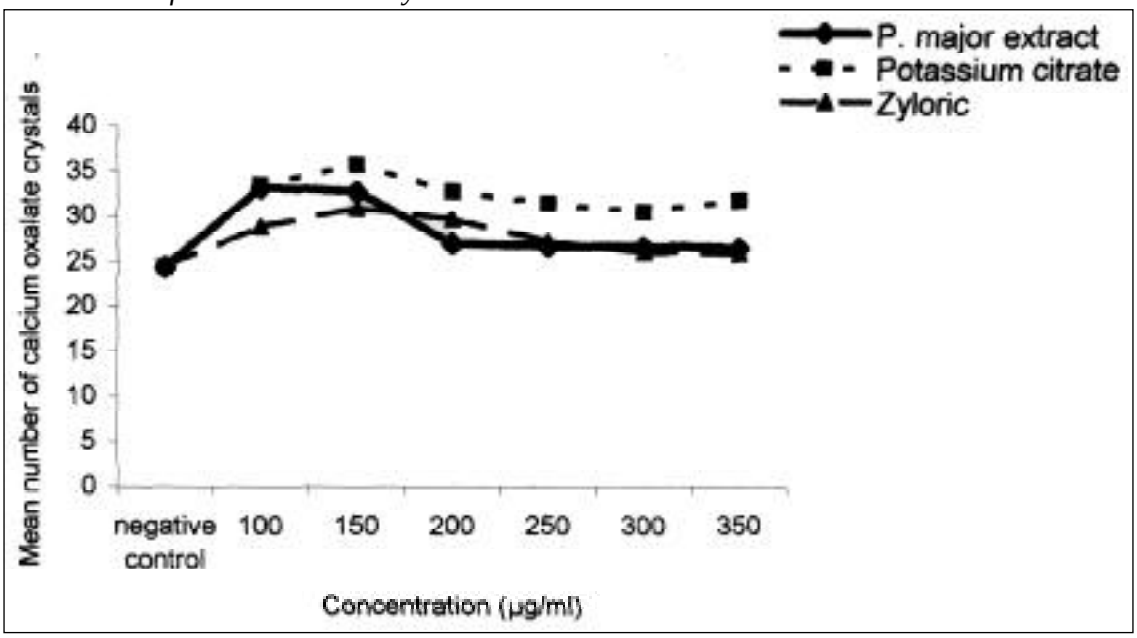


Figure 3 : Effects of Plantago major *extract, potassium citrate and allopurinol on the number of calcium oxalate crystals.*



equally divided into two areas. Eight equal wells with a distance of 1.25 x 0.5 cm were made on each slide when the gel was about to solidify. Calcium oxalate crystals were prepared by introducing equal amount of 20ul of 0.2M solution of calcium chloride and ammonium oxalate in the horizontal wells and sample and controls were put in the vertical wells. Modification of Schneider slide Gel method (1983) was used for this study and number and size of the crystal were measured after 24 hours by using Image analyser KS 300, 3.0 Carl Zeiss (11). The inhibition concentrations of *Plantago major* extract, allopurinol and potassium citrate were calculated and inhibition concentrations 50(IC_{50}) were determined for extract, allopurinol and potassium citrate (Fig. 1). Comparison between results of DMSO, *Plantago major*, allopurinol and potassium citrate were analysed using One way ANOVA (SPSS 11.0). Student 't' test (unpaired) was also used to compare results between any two groups. Level of significance was set at $p<0.05$.

Results

There was a white vertical line of formation of calcium oxalate crystals in each slide and the number of slides for each sample was twelve (n=12).

Table 1 : IC_{50} values of Plantago major *extract, potassium citrate and allopurinol on the size of calcium oxalate crystals* in vitro

	IC_{50} values	Mean size cystal (μm^2)
Negative control (DMSO)	-	128.50 ± 0.75
Extract of *Plantago major*	300 µg/ml	61.68 ± 0.42
Potassium citrate (positive control)	350 µg/ml	62.36 ± 0.15
Allopurinol (positive control)	450 µg/ml	63.70 ± 0.39

The measurement was done for three areas on each white vertical line for the number and size of the crystals by computerised Image Analyser and the mean ± SEM was calculated. There were significant reductions in the size of calcium oxalate crystals (dihydrate variety) by extract of *Plantago major* compared to control (99.9 ± 0.6μm²; 96.9 ± 0.5μm²; 91.0 ± 0.5μm²; 83.1 ± 0.5μm²; 61.9 ± 0.4μm²; 56.5 ± 0.4μm² vs 128.5 ± 0.8μm²) (*p<0.05) (n=60). Similarly the results for potassium citrate were (107.8 ± 0.4μm²; 105.0 ± 0.3μm²; 97.9 ± 0.2μm²; 91.3 ± 0.1μm²; 75.7 ± 0.2μm²; 62.4 ± 0.2μm² vs 128.5 ± 0.8 μm²) (*p<0.05) (n=60) and for allopurinol the results were (115.2 ± 0.5μm²; 107.2 ± 0.5μm²; 99.7 ± 0.4μm²; 92.3 ± 0.4μm²; 84.2 ± 0.4 μm²; 80.6 ± 0.3 μm² vs 128.5 ± 0.8 μm²). (*p<0.05) (n=60) (Fig. 2). The reduction on the number of calcium oxalate cryatals (dihydrate variety) for extract of *Plantago major* were (33.2 ± 2.6; 32.8 ± 3.2; 26.9 ± 1.3; 26.7 ± 2.2; 26.6 ± 3.2; 26.4 ± 1.9 vs 24.3 ± 1.5) but was not significant (p>0.05). Similarly the reduction on the number of crystals for potassium citrate were (33.4 ± 2.8; 35.7 ± 3.1; 32.8 ± 2.3; 31.4 ± 2.2; 30.5 ± 2.2; 31.8 ± 1.9 vs 24.3 ± 1.5) and for allopurinol were (28.8 ± 0.4; 30.8 ± 0.9; 29.7 ± 0.7; 27.2 ± 0.5; 26 ± 0.9; 25.8 ± 0.7 vs 24.3 ± 1.5). (Fig. 3). IC_{50} values (inhibition concentration 50) for the reduction on the size of crystals for *plantago major* was 300mg/ml, 350 for potassium citrate and 450 for allopurinol. (Table 1).

Discussion

The calcium oxalate crystals that have been produced in this *in vitro* study were similar to the crystals in the urine of patient with stone (12). Most of the crystals measured in this study were calcium oxalate (dihydrate variety) since 90% of monohydrate variety were formed only after 48 hours (13). *Plantago major* extract has signifcantly reduced the size of calcium oxalate crystals (dihydrate variety) (P<0.05). The higher the concentrations of extract the more will be the size reduction (Fig. 2). The active ingredients in the *Plantago major* are polysaccharides, fat, caffein acid derivatives, flavonoids, glycosides, irinoid and terpenoids (14). It may be one of the active ingredients that may have reduced the size of the crystals in this study. The mechanism of action was not known as yet. It may be the active ingredient in the ethanol extract of *Plantago major* which may have prevented the crystal formation or may have dissolved the preformed crystals (12). The action of potassium citrate may be that citrate may have combined with calcium ion to form calcium citrate so that there will be less chance of formation of calcium oxalate crystals (15). Since the core of calcium oxalate crystal contains 10-12 % of uric acid (9) allopurinol may have prevented the formation of calcium oxalate crystals by reducing the content of uric acid in the urine by acting through the enzyme xanthine oxidase (16). On the number of calcium oxalate crystals the lower concentrations of *Plantago major* up till 150mg/ml has no inhibition effect on the number of calcium oxalate crystals but the higher concentrations of *Plantago major* from 200mg/ml upwards do have inhibition effects but were not significant (p>0.05). According to the results obtained by IC_{50} on the size of the crystals, extract of *Plantago major* was the best inhibitor on the size of calcium oxalate crystals followed by potassium citrate and allopurinol.

Conclusion

Plantago major, potassium citrate and allopurinol do have significant inhibition effects on the size of calcium oxalate crystals (*p<0.05). All these three samples studied do have inhibition effects on the number of crystals but was not significant(p>0.05). According to IC_{50} values on the size of crystals, *Plantago major* was the best inhibitor on the size of crystals followed by potassium citrate and allopurinol.

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Malaysian Journal of Medical Sciences, Vol. 12, No. 2, July 2005 (27-33)

ORIGINAL ARTICLE

ABSENCE OF Ras, c-myc AND EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) MUTATIONS IN HUMAN GLIOMAS AND ITS CLINICAL FACTORS ASSOCIATED WITH PATHOLOGICAL GRADING AFTER SIX YEARS OF DIAGNOSIS IN NORTH EAST MALAYSIAN PATIENTS

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Neoplastic transformation appears to be a multi-step process in which the normal controls of cell proliferation and cell-cell interaction are lost, thus transforming normal cells into cancer. The tumorigenic process involves the interplay between oncogenes and tumour suppressor genes. In this study, we have selected the ras family, c-myc and epidermal growth factor receptor (EGFR) genes to detect whether their abnormalities are associated with the expression and progression of glioma cases in Malay patients. We have used the polymerase chain reaction-single stranded conformation polymorphism followed by direct sequencing for the study. For the ras gene family, we screened the point mutations in codons 12 and 61 of the H-, K-, and N- ras gene; for EGFR and c-myc, we analyzed only the exon 1 in glioma samples. In mutational screening analyses of the ras family, c-myc and EGFR gene, there was no mobility shift observed in any tumour analyzed. All patterns of single stranded conformation polymorphism (SSCP) band observed in tumour samples were normal compared to those in normal samples. The DNA sequencing results in all high-grade tumours showed that all base sequences were normal. All 48 patients survived after five years of treatment. In simple logistic regression analysis, variables which were found to be significant were hemiplegia (p=0.047) and response radiotherapy (p=0.003). Hemiplegics were 25 times more likely to have high pathological grade compared to those without. Patients with vascular involvement were 5.5 times more likely to have higher pathological grade. However, these findings were not significant in multivariate analysis. Patients who had radiotherapy were nearly 14 times more likely to have higher pathological grade. Multivariate analysis revealed that patients with hemiplegia were more likely to have higher pathological grade (p= 0.008). Those with higher pathological grading were 80 times more likely to have radiotherapy (p=0.004).

***Key words** : Ras gene, c-myc, EGFR, Gliomas, Malaysia*

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Introduction

Neurological tumours are common neoplasms of both adults and children. Recent studies have attempted to delineate the genetic abnormalities that underlie such tumours, and have implicated two classes of genes, oncogenes and tumour suppressor genes. Neoplastic transformation also appears to be

a multi-step process in which the normal controls of cell proliferation and cell-cell interaction are lost, thus transforming a normal cell into a tumour cell. This tumorigenic process involves the interplay between at least two classes of genes: oncogenes and tumor suppressor genes. Oncogenes are abnormally activated versions of cellular genes that promote cell proliferation and growth. Therefore, activated oncogenes result in an exaggerated impulse for a cell to grow and divide.

Gliomas mostly occur sporadically, and are known to be non-inherited tumours. The predisposition to develop gliomas is also associated with hereditary illness (including NF-1), tuberculosis complex, Gardner's syndrome, Turcot's syndrome, Li-fraumeni syndrome and other factors. Binger and colleagues have suggested that despite the heterogeneity observed among glioblastoma karyotypes, several distinct abnormalities are frequently detected as losses of chromosome 10 (60%) and 17, gains of chromosome 7 (18%), translocation and deletions of chromosome 9p at lower frequency (20%) and losses of chromosome 22 and sex chromosome (http://www.thamburaj.com/neurogenetics.htm).

Molecular abnormalities associated with primary brain tumours include a wide variety of changes in tumour suppressor genes, proto-oncogenes and growth factors.

Ras gene families consist of 3 members: N-*ras*, H-*ras* and K-*ras*, that encode for the highly homologous protein called p21 according to their molecular weights. The inactivation of ras genes by point mutation is the most frequent and well known genetic alteration associated with human cancer including brain tumours. Common mechanisms of inactivation of these genes include missense mutations at the well known hot spot of codon 12, 13 and 16 (1).

The presence of *ras* mutation has also been shown to be significant for prognosis and considering the importance of the *ras* gene in tumorigenesis, this gene might be a good target for the development of anti-cancer therapy (2).

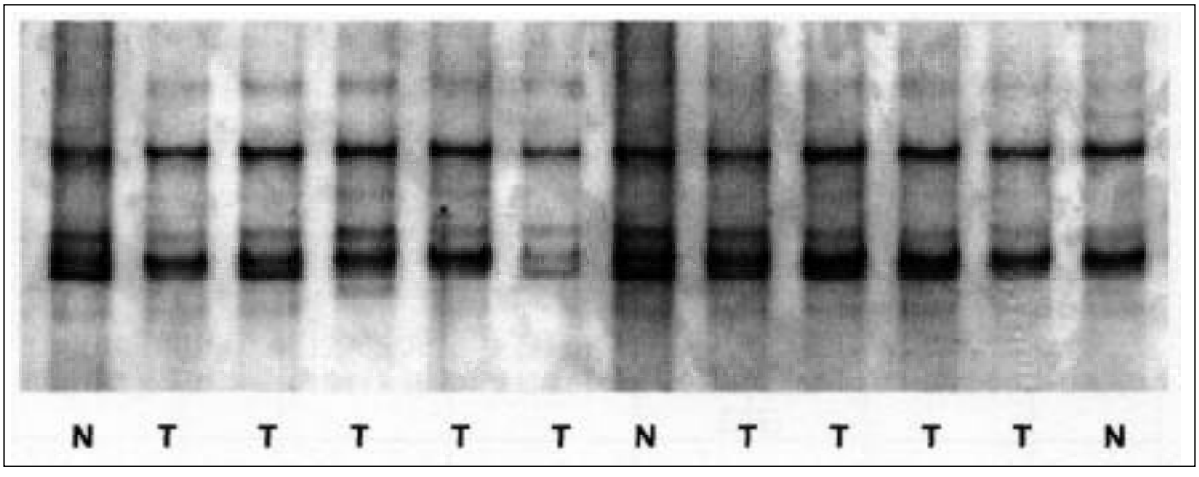
The c-myc gene, mapped on human chromosome 8q24, encodes the transcription factor c-myc, that heterodimerizes with a partner protein, Max, to regulate gene expression. The amplification of the c-myc gene in neuroblastomas appears to correlate with the clinical stage of the tumour and poor prognosis. Therefore, it is tempting to speculate that the c-myc protein is one of the essential products necessary for the aberrant behaviour of neuroblastoma cells (3).

Over-expression and amplification of c-myc may play an important role in metastatic progression, and there is evidence that it indicates poor prognosis in the largest clinical populations of breast, colon, lung and pancreatic cancers (3).

c-myc's status as an important target was reinforced by a recent widely-reported paper published in *Science* by Dean Felsher at Stanford, in which he demonstrated that a temporary reduction in c-myc expression followed by reactivation, in a genetically engineered mouse model, induced highly selective and complete apoptosis in cancer cells, while having no effect on normal cells (4).

The EGFR gene is a multifocal allosteric transmembrane protein with an intra cellular binding site for EGF, and acts as a tyrosine kinase. The gene is localized on the short arm of chromosome 7, within 7p11 – 13, and is called erb – B1. This

*Figure 1: Polymerase chain reaction-assisted single-strand conformation polymorphism (*PCR-SSCP*) analysis of N-ras gene in glioma. N represents the normal sample and T, the glioma sample*



receptor has been found to be over-expressed in 50% to 70% of glioblastoma multiforme. Although the functions of the proteins encoded by most proto-oncogenes are not precisely known, biochemical activities of several proto-oncogene products have been identified. Some of the gene products are identical or related to proteins known to be important in growth regulation.

We analysed ras family, c-myc and EGFR to determine whether they are involved in the tumourigenesis of gliomas in a group of patients in Hospital Universiti Sains Malaysia in North East Malaysia. The objective of this study was to relate the mutation analysis to the eventual response of these tumours to clinical outcomes.

Materials and Methods

Sample Collection and DNA Extraction

A series of 41 glioma specimens were obtained from the Brain Tumour Bank of the Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia with ethical approval of the Research and Ethics Committee, Universiti Sains Malaysia. The tumours were classified according to the World Health Organizations (WHO) classification. DNA was extracted from the tumour tissue using commercial extraction kits (QIAGEN Inc. USA).

PCR Amplification

PCR was performed to amplify DNA segments of H-ras, K-ras and N-ras. The nucleotide sequences of H-ras, K-ras and N-ras were shown in Table 1 (1). PCR reactions were performed in 100 µl volumes using 50 to 100 ng of genomic DNA templates, 1 X PCR buffer, 200 µM of dNTP, 2 mM $MgCl_2$, 1.0 µM (50 pmol) of each primer and 2.5 unit of Taq DNA polymerase (Fermentas USA). Thirty-five cycles were performed as follows: denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. after the last cycle of amplification, the extensions were continued for an additional 7 min at 72°C.

Primers designed to amplify the c-myc exon I intron I regulatory region contained the sequence in Table 2. The PCR products were 589 bp in size and included 162 bp in exon I and 427 in intron 1. The PCR reaction mix consists of 1 µl of DNA extract, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM

Table 1 : Simple logistic regression showing factors associated with pathological grading amongst patients with human gliomas

Variable	Pathological gradings Low grade n (%)	High grade n (%)	Crude odds ratio (95% CI)	LR* statistic	p-value
Age (yr)					
≤ 40	13 (65.0)	7 (35.0)	1	-	-
> 40	15 (71.4)	6 (28.6)	0.74 (0.20, 2.78)	0.20	0.659
Sex					
Male	15 (65.2)	8 (34.8)	1	-	-
Female	13 (72.2)	5 (27.8)	0.72 (0.19, 2.76)	0.23	0.6333
Epilepsy					
Yes	13 (65.0)	7 (35.0)	1.35 (0.36, 5.04)	0.20	0.659
No	15 (71.4)	6 (28.6)	1	-	-
Hemiplegia					
Yes	9 (42.9)	12 (57.1)	25.29 (2.84, 225.43)	14.60	0.004
No	19 (95.0)	1 (5.0)	1	-	-
Vascular					
Yes	14 (56.0)	11 (44.0)	5.50 (1.03,29.48)	4.87	0.047
No	14 (87.5)	2 (12.5)	1	-	-
Radiotherapy					
Yes	8 (42.1)	11 (57.9)	13.75 (2.47,76.42)	11.95	0.003
No	20 (90.9)	2 (9.1)	1	-	-

* LR statistic – likelihood ratio statistic

$MgCl_2$, 200 µM of each dNTP (Promega, Southampton, U.K) in a total volume of 25 µl. Forty cycles of PCR were carried out on a thermal cycler (Perkin Elmer) consisting of denaturing at 94°C for 30 s, annealing at 65°C for 30s and extension at 72°C for 45°C. And initial denaturing step at 95°C for 5 min preceded the addition of enzyme and an extension step at 72°C for 5 min concluded the reaction. Three microlitres of PCR product was checked for yield size on a 1% agarose gel. A total of 5 µl of PCR products was digested by Taq I (Promega Inc. USA) to generate one 223 bp and two 183 bp fragments.

The total volume of the PCR mixture of EGFR was 100 ml containing 1X PCR buffer, 200 mM of dNTP, 2 mM $MgCl_2$, 1.0 mM (50 pmol) of each primer and 2.5 unit of Taq DNA polymerase (Fermentas USA). Amplification was carried out in MJ Research for 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with an initial denaturation step at 94°C for 10 min, and a final extension at 72°C for 10 min, for 30 cycles.

SSCP (Single-Strand Conformation Polymorphism)

Each of 8 µl PCR product of ras, c-myc and EGFR were added to 4 µl of sequencing stop solution (Amresco Inc. USA). The mixture was heated at 90°C for 5 minutes, chilled on ice and applied to a 0.5X MDE gel (FMC Bioproduct USA) or 6% acrylamide gel containing 5%-10% glycerol. Electrophorisis was performed using the Dcode universal mutation Detection System (Bio-Rad Laboratories USA) at 10 watts constant and at room temperature. The running time was approximately 14 to 18 hours. The gels were stained using silver-staining (5).

Direct Sequencing Analysis

The samples that showed abnormal mobility in the SSCP analysis were isolated and run on 2% agarose gels and purified using a Geneclean II kit (Bio101 Corp, USA) in preparation for sequencing. Purified products were sequenced on an ABI 3100 automatic sequencer using the same primer as those used in the PCR-SSCP.

Clinical, Radiological and Treatment Parameters

The patients were studied over a period of 60 months. Parameters like age, sex, socio- economic status, and presenting complaints such as headache, visual disturbances, hemiplegia, epilepsy as well as radiological images (site, size, consistency of tumour, oedema, calcification, haemorrhage, intensity on MRI, midline shift and vascularity) were compared to their management, ras family, c-myc and EGFR mutations, relapse rate and outcome. Socio-economic groups were divided into upper class (> 250 USD), middle class (100-250 USD) and lower class (<100 USD).

Table 2 : Multiple logistic regression analysis showing factors associated with pathological grading amongst patients with human gliomas

Variable	Adjusted odds ratio (95% CI)	Wald statistic	p-value
Epilepsy			
No	1	-	-
Yes	0.12 (0.01, 2.16)	2.06	0.152
Hemiplegia			
No	1	-	-
Yes	171.36 (3.88, 7566.01)	7.09	0.008
Vascular			
No	1	-	-
Yes	5.78 (0.31, 106.84)	1.39	0.239
Radiotherapy			
No	1	-	-
Yes	80.92 (3.94, 1662.91)	8.12	0.004

Hosmer & Lemeshow test X^2=9.19, p=0.24, classification overall percentage=90.2%.

All patients underwent a MRI GE 1 Tesla prior to surgery and received gadolinium following certain protocols. Location of tumours was recorded according to the respective lobes and sites.

Size of tumours was measured using the standard software provided by the GE MRI machine. The consistency of tumours were reported by two blinded radiologists as defined by a Japanese study (2). Calcification was detected by CT Scan of the brain with a Hounsfied between 100-300 H.U. . Haemorrhage in the tumour is defined as any hyperdensity (Hounsfield between 75 to 80 H.U.) measured using the classical protocol. Midline shift was defined as any deviation of the midline, taking the pineal gland as the centre.

Vascularity was defined as any tumour blush seen on MRI done on the 1 Tesla machine as seen by two blinded neuroradiologists. All patients underwent total removal of tumours confirmed by repeat CT scans with contrast within 24 hours of operation. If the tumour was still present a re-operation was done to remove all tumour tissues and reconfirmed by CT scan of the brain with contrast. Treatment modalities were chemotherapy, radiotherapy and immunotherapy as requested and agreed by both radiation therapist/oncologist and patient. Recurrence on CT/MRI was defined as any disease or lesion returning or showing a tendency to return from time to time within the study period. Relapse of signs and symptoms were defined as the reoccurrences of symptoms similar to the previous complaints or signs seen again on physical examination without the knowledge of the radiological results.

Ras family, c-myc and EGFR mutations were examined using various methods mentioned above. Outcome was defined as alive in good condition with a score of > 70, alive in poor condition, being unable to fend for oneself completely with a Karnofsky score of < 70 and dead.

All patients were operated on and had a Karnofsky score of more than 70 before being included in the study. All histopathological examinations were reported according to the WHO classification and seen by at least three histopathologists and discussed in our neuropathology conferences and all the specimens were analysed twice to rule out false or negative results.

Statistical Analysis on Factors Associated with Pathological Gradings

Simple logistic regression analysis was applied to determine potential factors associated with the pathological grading. Pathological grading was treated as a binary outcome variable. Crude odds ratios with 95% confidence intervals likelihood ratio statistics and corresponding p-value were presented. Variables that were significant in simple logistic regression and variables that were considered to be clinically important were included in multivariate analysis.

Multiple logistic regression analysis was applied to determine factors associated with the pathological grading. Stepwise backward logistic regression was used with the probability of entry at 0.05 and removal at 0.3. Likelihood ratio test was applied in the modeling procedure. Adjusted odds ratios with 95% confidence interval, Wald statistic and corresponding p-values were presented. Fit of the model was determined by using the Hosmer and Lemeshow test and overall classification percentages.

Results

Identification of Mutation of Ras Family, c-myc and EGFR Gene

Forty one glioma samples underwent PCR amplification by using H-ras, K-ras N-ras, c-myc and EGFR gene primers and were subjected to PCR-SSCP analyses and DNA sequencing. In mutational screening analyses of the ras family, c-myc and EGFR genes, there was no mobility shift observed in any tumour analyzed. All patterns of SSCP band observed in tumour samples were normal compared to those in normal samples. The DNA sequencing results in all high-grade tumours showed that all base sequences were normal.

Clinical Factors Associated with Pathological Findings

All 41 patients survived after six years of treatment. In simple logistic regression analysis, variables which were found to be significant were hemiplegia (crude OR 23.29, 95% CI 2.84- 225.45, p=0.004), vascularity (crude OR = 5.5, 95% CI 1.03 – 29.48, p=0.047) and response radiotherapy (crude OR=13.75, 95% CI 2.47-76.42, p=0.003). Hemiplegics were 25 times more likely to have high pathological grades compared to those without. Patients with vascular involvement were 5.5 times more likely to have higher pathological grades. However, this finding was not significant in multivariate analysis. Patients who had radiotherapy were nearly 14 times more likely to have higher

pathological grades.

Multivariate analysis revealed that patients with hemiplegia were more likely to have higher pathological grades (adjusted OR =171.36, 95% CI 3.88 – 7566.01, p= 0.008). Those with higher pathological gradings were 80 times more likely to have radiotherapy (adjusted OR 80.92, 95% CI 3.94-1662.91, p=0.004).

Discussion

There were no mutations of 3 types of genes (ras family, c-myc and EGFR) in our study. We identified no abnormal SSCP band shift in these samples and reconfirmed by performing direct DNA sequencing analysis.

The ras family, c-myc and EGFR genes were not involved in the tumourigenesis in our patients and may not be the common inactivation mechanism in the pathogenesis of gliomas in North East Malaysian patients.

The authors of previous studies have also shown that EGFR gene was often mutated in high-grade gliomas in adult, but the frequency of EGFR mutations was still low (6, 7). Feng et al (8) reported that 90% of human pancreatic cancers, 50% of colon cancers, and morethan 30% of smoking-related lung cancers have a mutation at codon 12 of the K-ras gene. Interestingly, only 5% of lung cancers that are not smoking-related containa mutation at codon 12 of the K-ras gene. The presence of ras mutation has also been shown to be significant for prognosis and considering the importance of the ras genes in tumorigenesis, the ras gene might be a good target for the development of anti-cancer therapy (8). However, our results revealed that no mutations were present in the ras gene family in gliomas using the Polymerase Chain Reaction- Single Strand Confirmation Polymorphism (PCR-SSCP) analyses in the DNA extracted from glioma tissues.

According to Yusoff et al, Zan et al and Ghazali et al, there were no ras mutation detected in all tumours analyzed from Malaysian patients (9 - 11). They concluded that this gene does not play a major role in the tumourigenesis of malignant gliomas (6). Direct sequencing analysis in oral tumours also reported that no mutation has been found in N-ras, K-ras and H-ras genes. Activating ras mutations can be found in human malignancies with an overall frequency of 15-20%. A high incidence of ras gene mutations has been reported in malignant tumours of the pancreas (80-90%, K-ras), colorectal carcinomas (30-60%), non-melanoma skin cancer (30-50%, H-ras), hematopoietic neoplasia of myeloid origin (18-30%, K-and N-ras) and seminoma (25-40%, K-ras). In other tumours, a mutant ras gene is found at a lower frequency: breast carcinoma (0-12%, K-ras), glioblastoma and neuroblastoma (0-10%, K-and N-ras) (8).

Trent et al described the amplification and expression of the cellular oncogene c-myc in double-minute-containing cells from one patient with glioblastoma multiforme, and they have shown that the amplification is associated with the rearrangement of the c-myc gene (12). This finding further supports the uncommon association of the myc gene family in neurogenic tumours and provides rare evidence of myc gene amplification in high grade gliomas. In another study, c-myc gene was involved in leukemia, breast, stomach and lung cancer.

The c-myc gene was discovered as the cellular homolog of the retroviral V-myc oncogene 20 years ago. The c-myc proto-oncogene was subsequently found to be activated in various animal and human tumours . It belongs to the family of myc gene that includes B-myc, L-myc, N-myc and s-myc; however only c-myc, L-myc and N-myc have neoplastic potentials.

It is possible that other mechanisms exist where ras family, c-myc and EGFR inactivation are involved in the development of brain tumours in our Malay patients.

It is interesting to note that these patients are still alive 6 years after treatment despite some having high grade gliomas.

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ORIGINAL ARTICLE

AMBULANCE SERVICES AT HOSPITAL UNIVERSITI SAINS MALAYSIA AND HOSPITAL KOTA BHARU: A RETROSPECTIVE STUDY OF CALLS

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This retrospective study attempted to identify the pattern of ambulance calls for the past two years at the Hospital Universiti Sains Malaysia (HUSM) and Hospital Kota Bharu (HKB). This study will provide a simple method of acquiring information related to ambulance response time (ART) and to test whether it met the international standards and needs of the client. Additionally, this paper takes into account the management of emergency calls. This included ambulance response time, which was part of Emergency Medical Services (EMS) episode: onset of ART, which started when details like phone number of the caller, exact location of the incident and the nature of the main complaint had been noted. ART ended when the emergency team arrived at the scene of incident. Information regarding ambulance calls from the record offices of HUSM and HKB was recorded for the year 2001 and 2002, tabulated and analyzed. There was a significant difference in the total number of calls managed by HUSM and HKB in the year 2001. It was noted that 645 calls were managed by HUSM while 1069 calls were recorded at HKB. In the year 2002, however, HUSM led with 613 extra numbers of calls as compare to HKB with 1193 numbers of calls. The pattern of ambulance calls observed is thought to possibly be influenced by social activities like local festivities, school holidays and the seasons. Further, it is observed that no studies were previously undertaken to compare the ART at both the HUSM and HKB to that of the international standards. In fact, a literature review undertaken so far showed no similar studies have been done for the whole Malaysia.

***Key words** : Emergency Medical Services, Emergency Ambulance Services, Calls.*

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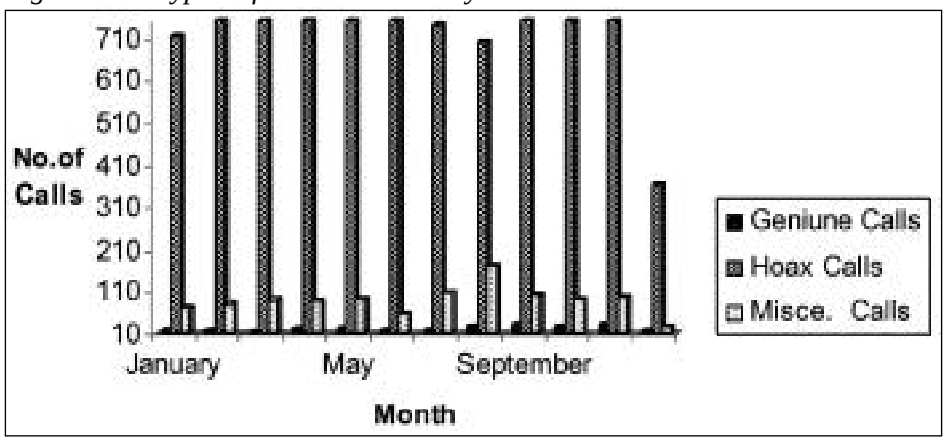
Introduction

Emergency Medical Services (EMS) system comprises of a comprehensive network of personals, equipment and resources established with the aim of delivering aid and emergency medical care to the community. These services are meant also to ensure that deliveries of care are partaken rapidly, effectively and with quality assurance. EMS efficiency can be measured in many ways. The most important way of looking at this system is by examining the whole process of EMS episode as mentioned by Guppy & Wollard (1).

Meislin *et al., (*1999*)* defined the components of EMS episode based on timing as consisting of the following; receiving of a call, call processing time, control allocation time, crew mobilization time, traveling time to the scene, traveling time to

Figure 1: Types Of Calls Received By Rescue 991 In The Year 2001



the Emergency Department (ED) or hospital and ends with time spent at ED or hospital (2). In fact, the whole process of EMS episode plays a crucial role in determining service output. The EMS episode consists of 7 distinct period mentioned above with a component named 'response time' consisting of 3 components namely, control allocation time, crew mobilization time and traveling time to scene (3). The crucial determinant of successful EMS episode can be illustrated clearly with cases of out of hospital cardiac arrest patients. The concept that time interval between getting early initial treatment at the so-call 'golden hour' phase provided by ambulance crews has a greater effect on the morbidity and mortality rate of the victim or patient. A North American study stated that every minute delay in the initiation of cardio pulmonary resuscitation (CPR) during cardiac arrest for example, could increase the mortality and morbidity rate by up to 7 to 10% (4).

Ambulance response time is undoubtly one of the parameters usually used in measuring EMS efficiency. Pell *et al.*,(2001) stated that in the United Kingdom, ambulances are expected to arrive at the designated scene within 7 to 14 minutes for 90% of the calls received (5). In addition, Breen *et al.*, (2000) found that 14% of calls took 5 minutes or longer to activate response while 38% of emergencies obtained responses within 9 minutes. Further, it was noted that only 4.5% of emergency calls originating from places greater than 5 miles from the ambulance bases responded to within 9 minutes (6).

Narad & Driesbock (1999), in their study on all California local EMS agencies had shown that only 57% of Californian counties ambulance services regulate their response times. Many of the ambulance enforcement programs in California have enforcement mechanism that is unlikely to promote compliance. Therefore, response time regulation is intended to improve the effectiveness of the EMS system in pre hospital care (7).

Studies done in Perth, Australia shows similar finding. It was found that the trend in occurrence and survival following out of hospital cardiac arrest are similar to those found elsewhere (8). Another study was also done with the objective to determine the relative effectiveness of differences in response

Figure 2: Genuine Calls Received By The Different Shifts At ED Of HUSM In The Year 2001

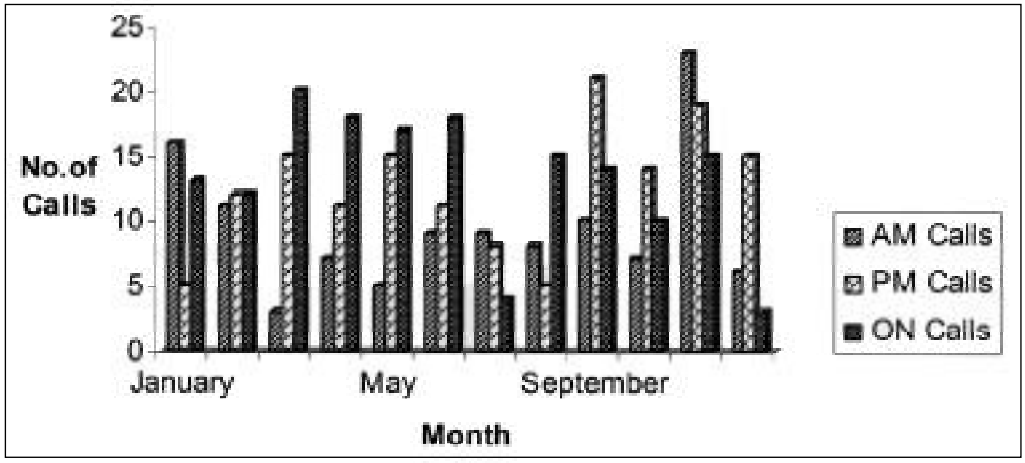
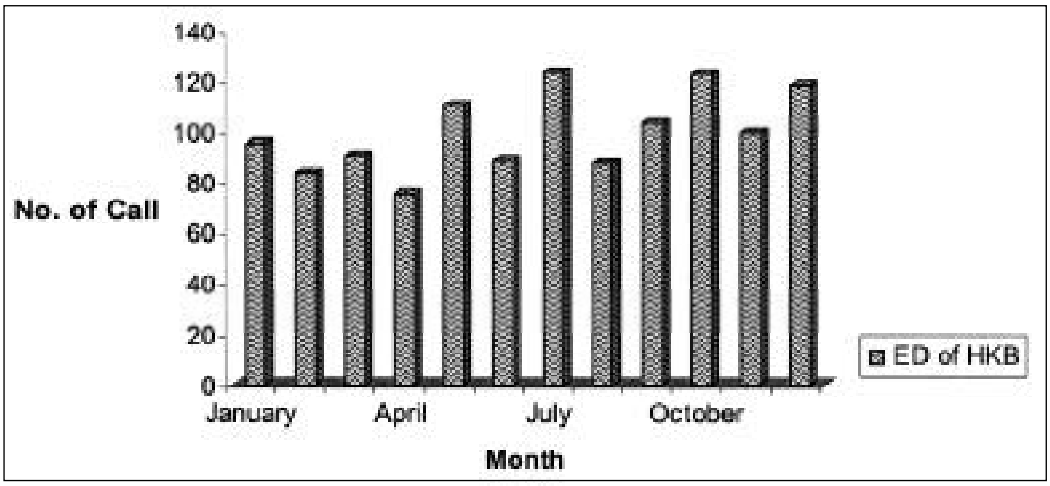


Figure 3: Number Of Calls Received By ED Of HKB In The Year 2001



time interval, proportion of bystander Cardiopulmonary Resuscitation (CPR) and type and tier of EMS system on survival after out of hospital cardiac arrest (9). They concluded that increased survival rates of these patients upon discharge from the hospital might be associated with decreased response time interval and the used of two tier EMS systems as compared to a one-tier system.

Another call-response interval study was done in the Turkish city of Ankara with the aim of trying to determine the various times related to the ambulance activities of Ankara Emergency Aid and Rescue Services (EARS) (10, 11). These authors expected that results of their studies might contribute to the improvement of the EMS system. A descriptive study was planned to determine various times related to the ambulance activities of Ankara EARS. The variables of the study were: delay time, response time, time at the scene (scene time), round trip time, transport time and total run time of Ankara EARS ambulance activities. The median response time of Ankara EARS was found to be 9 minutes. In the research year, the median delay time was 2 minutes. Median arrival time to patient contact time of Ankara EARS was 2 minutes. Median time at the scene was 7 minutes. Median round trip time of the system was 44 minutes. The median time for the arrival at the scene from the ambulance station was 8 minutes. The median transport time was 10 minutes. The median total run time was 30 minutes. As the median response time was found to be 9 minutes it is concluded that there should be more ambulance vehicle to improve this response time for Ankara EARS. It is observed, that due to financial problems the ambulance crew and dispatchers of Ankara EARS recorded their times manually (10). These authors also concluded that if digital and electronics recording systems were utilized, time recordings could be more precise.

Based on a report commissioned by The Ministry of Health and Social Affairs of Norway, Steen-Hansen *et al.*, (2000), proposed that standards for ambulance response intervals in emergencies if performed would be able to assess the current

Figure 4: Comparison Of The Number Of Genuine Calls Managed By HUSM And HKB In The Year 2001

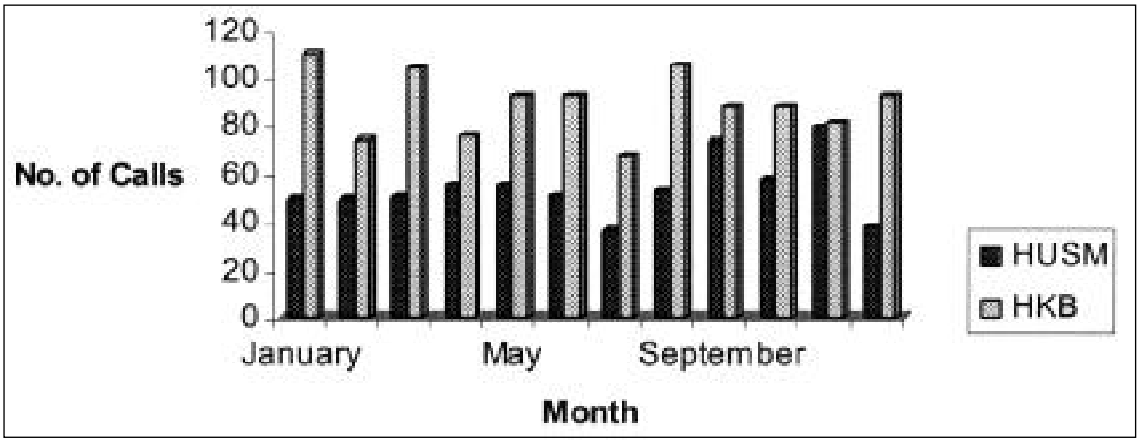
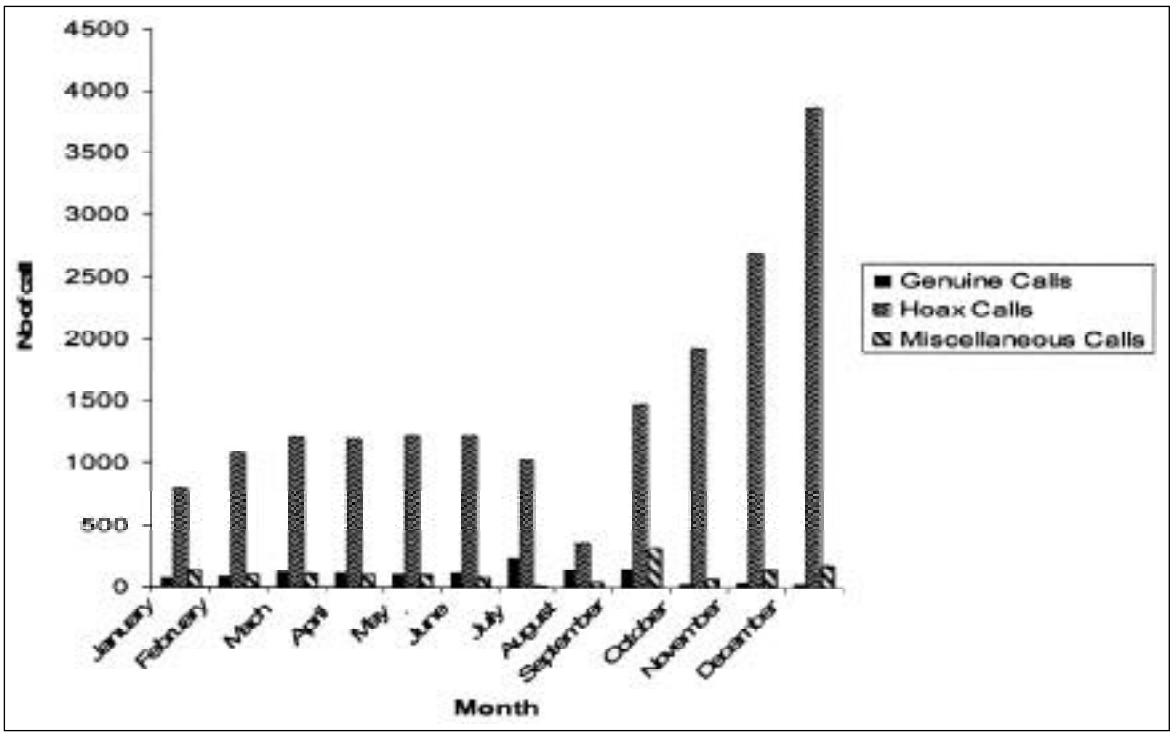


Figure 5: Type Of Calls Received By Rescue 991 In The Year 2002



ambulance response interval (12). The report also proposed that an ambulance should reach 90% of the population in cities and urban areas within 8 minutes, whilst in rural areas, 90% should be reached within 25 minutes. The study concluded that the proposed standard was not achieved in any of the municipality in the country. However, the city of Tonsberg having shown the best performance can only achieved 48.9% of the populations where ambulance reaches their destination within 8 minutes.

In Singapore, a study conducted just over a decade ago had ascertained the time it took for ambulance team to reach a patient and transport the patient to an emergency department after an emergency call (13, 14). It was reported that it took an average of 11.40 ± 4.88 minutes for an ambulance team to reach a patient and 30.50 ± 10.62 minutes for the patient to reach an emergency department. At the level of staffing in Singapore at that time, the basic life support care starts around 11.40 minutes whilst advanced life support care started 30.50 minutes after an emergency call.

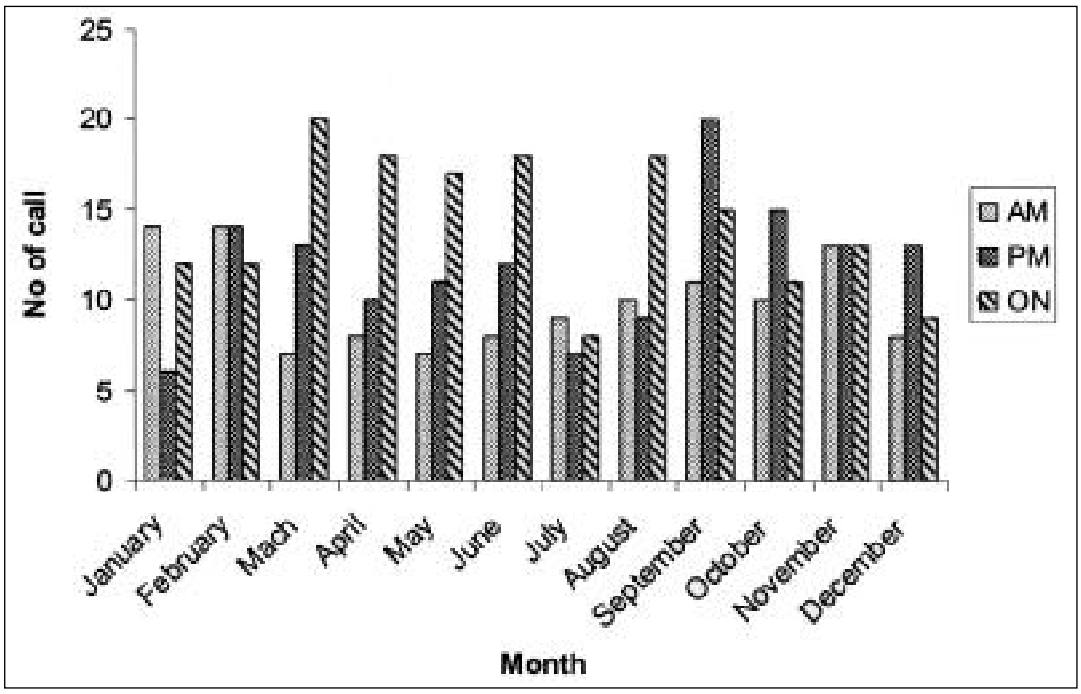
In Malaysia, no published studies related to ambulance services and their efficiency was found so far. It is reasonable, therefore, to conclude that any study in Malaysia would be novel. Like other developing countries, the Malaysian government subsidizes a substantial portion of the healthcare cost. There is, however, a lack of effort and money spend towards the development of pre hospital care component such as ambulance services as compared to expenditure and concern given to the public health and in-patient care. Throughout Malaysia, the government through Ministry of Health is the main provider of ambulance services. These ambulance services are hospital based and manned usually by a driver, hospital attendant and assistant nurse or staff nurse or medical assistant.

In light of the above situation, it was thought that a retrospective study on ambulance calls in the Emergency Department of Hospital Universiti Sains Malaysia (HUSM) and Hospital Kota Bharu (HKB) would be appropriate as a pilot study. The need for this kind of studies was thought to be long overdue since the HKB ambulance services started in 1960, while HUSM since the hospital started services in 1983. No formal study was done at these places to either determine the efficiency or the extended services of the EMS in Kota Bharu region. The present study is an attempt at collecting and analyzing data recorded at both hospitals over a period of two years (2001 and 2002). Results obtained may then provide background information for further studies.

Methods

Records at the Emergency Departments of HKB and HUSM were collected and discussion with ED staff at the both places were undertaken. The information gathered from these discussions together

Figure 6: Total Number Of Calls Received At Different Shift By ED Of HUSM In The Year 2002



with recorded data were collated and analysed.

Since the year 2000, a squad called Rescue 991 under the Jabatan Pertahanan Awam Malaysia (A special government body established to assist in all emergency and disaster event in Malaysia) was located at ED of HUSM. Their specific aim is to help extend social work services to the public including ambulance services. These services include emergency cases or non-emergency cases. This arrangement is unique and only exists in HUSM, whereas, in HKB these kinds of services were purely undertaken by the hospital staff.

Information gathered from HUSM must therefore be combined together with some record from Rescue 991 unit. All data from the records office at HUSM and HKB were collected loaded to the computer and analyzed using Microsoft Excel,. Information regarding ambulance calls recorded were for the year 2001 and 2002.

Results

Record for the year 2001

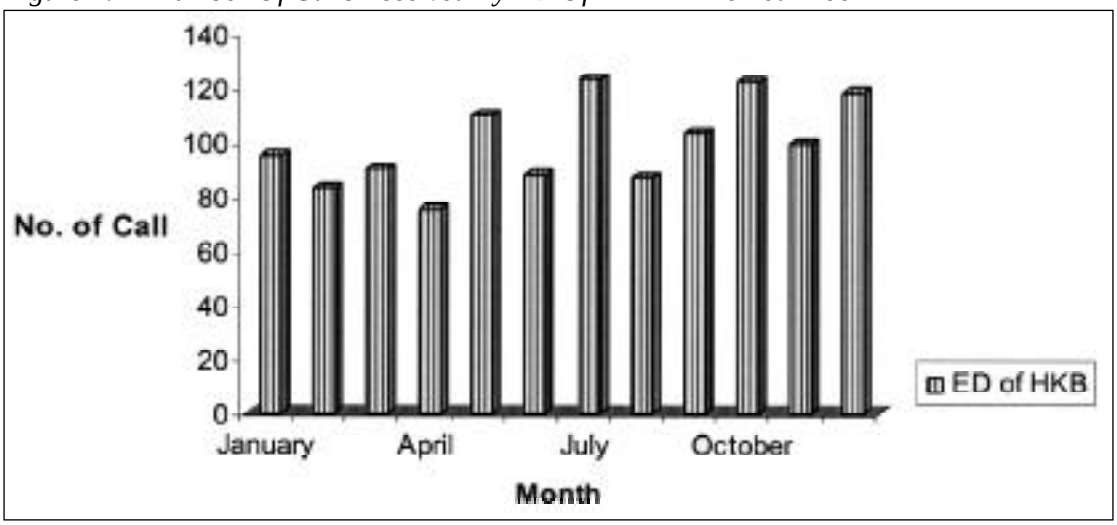
Upon examination of previous recorded data at the Emergency Departments (ED) of HUSM and HKB, a retrospective study on ambulance calls was performed. At the HUSM, two groups of data were collected - from Rescue 991 records and from records of ED of HUSM. In 2001, Rescue 991 received a total of 10,170 of calls. Out of the total number of calls, there were 231 genuine calls while a whooping 8871 calls were hoax and the rest were categorized as miscellaneous calls

The numbers of genuine calls were noted to be lower in the months of December to July but were almost double in the months of August to November. Whereas, hoax calls were consistent for the whole year. April, September and November were the peaks months. This correspond to the school holiday period and may explain the high number of calls. July to September was the peak in the miscellaneous call category (Figure 1a)

In ED of HUSM, a total 338 of calls were noted and managed subsequently by them in the same year. This information is shown as divided shifts. The morning shift (7am to 2pm) shows the highest number of calls in November while March shows the lowest. The afternoon shift (2pm to 9pm) registers the lowest number of calls in January and August while the highest was in September. Lastly, the night shift (9pm to 7am) shows that the highest numbers of calls were in March to June while December is the month that received the lowest number of calls. (Figure 2a). It can be concluded that there were not many differences in term of the number of calls for the whole year.

The ED of HKB on the other hand managed to receive a total of 1069 ambulance calls in 2001. The months of January, March and August had a total number of calls exceeding more than 100. The highest numbers of calls were in January with 110 calls, followed by August with 105 and March with

Figure 7: Number Of Calls Received By ED Of HKB In The Year 2002



104. The lowest number of calls for year 2001 was in July with only 67 calls.

Four peaks were seen in the yearly tabulation of the number of calls. This was seen in January, March, May, June, August and December while July recorded the lowest number of calls (Refer figure 3a).

There was a large difference in the total number of calls managed by HUSM and HKB in the year 2001. 645 calls were managed by HUSM while 1069 calls were recorded at HKB. HKB consistently managed a higher proportion of calls in almost every month, except for November (Figure 4a).

Records for the years 2002

In a year 2002, the data was different especially in a total number of calls. The total number of calls received by Rescue 991 increased to 20669. Out of the total calls only 1254 were a genuine calls while a whooping 18039 calls were hoax and the rest were classifies as miscellaneous.

The number of genuine calls was noted to be higher in the months of March to September with peak in July but troughs in October to December. Monthly total numbers of hoax calls were more than one thousand except in the months of August (356 calls) and January (795 calls). Miscellaneous calls registered saw July and December as the months with the lowest number of calls at 7 and 179 cases respectively while the highest was December with 179 cases (Figure 5a).

ED of HUSM managed to receive 552 calls in 2002. This is tabled accordingly with shifts. The morning shift shows the lowest number of calls in March and May while January and February show the highest. Afternoon shift shows that monthly total number of calls exceeded 10 except for the month of January, July and August. Night shift calls show peaks in March and become lowest in July (Refer figure 6a).

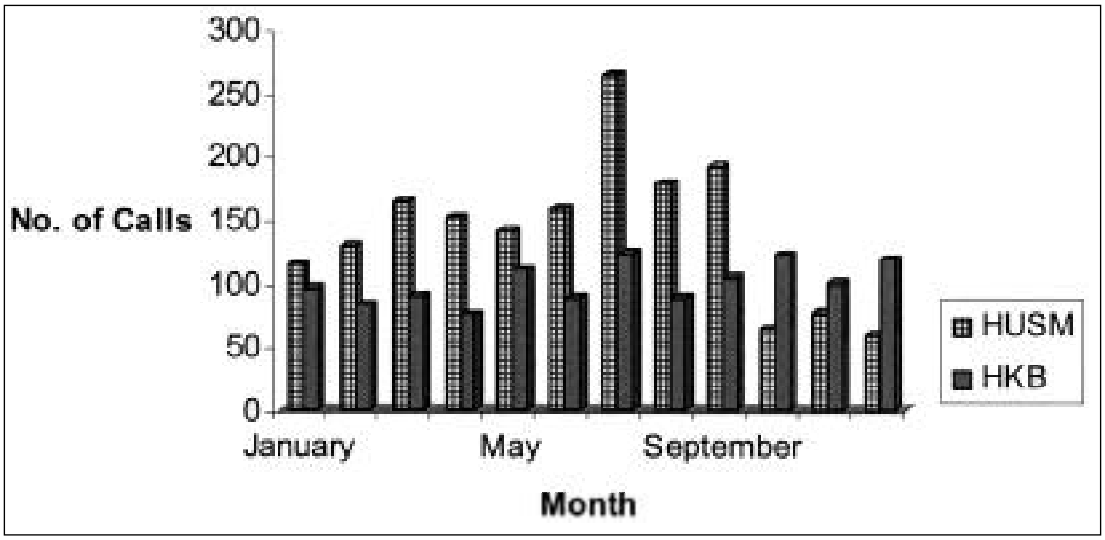
ED of HKB received a total 1193 number of calls. The months of May, July, September, October and December recorded more than 100 numbers of calls with the highest being in July (123). April is the month with the lowest number of calls (75). Figure 7a shows that there were several peaks in the number of calls for the year 2002. They were in January, May, July, October and December, while April registered the lowest.

There were obvious differences between the activities at the HUSM and HKB. HUSM received 613 numbers of calls, which is more than HKB for that year. The month of July received the highest number of calls at both hospitals but the lowest for HKB was in April whereas for HUSM it was in October (Figure 8a).

Discussion

Information regarding ambulance calls recorded for the year 2001 and 2002 at both hospitals; HUSM and HKB show that there was an increase in the percentage of calls for both the hospitals in a year 2002. HKB experienced an 11.6 % increase in the number of calls whilst HUSM showed an increase of 180%. The huge increase in HUSM figure is explained by the existence of the special squad called Rescue 991 under the Malaysia Department of Civil Defense (A special government body established to assist in all emergency and disaster events in Malaysia) was located at HUSM ED since 2000. Their specific aim is to help extend social work and services to the public including

Figure 8: Comparison Of The Proportion Of Genuine Calls Managed By HUSM And HKB In The Year 2002



ambulance services. This arrangement is unique and only exists in HUSM, whereas, in the HKB this services were purely undertaken by the hospital staff. Information gathered from HUSM must therefore be combined together with some record from Rescue 991 unit

Apart from the increased in the percentage of the number of calls for the year 2002, the result of this study also showed that there were peculiar pattern of calls at both hospitals. The pattern of ambulance calls at the both places; HUSM and HKB looks similar and it was observed that it might be due to the yearly social activities like festivities, school holidays and climatic seasons. For an example, in the year 2001, the total number of calls was increased in February and March. These periods coincide with the celebration of the *Hari Raya* for Muslims and the Chinese New Year for the Chinese. In the months of September to December, the same pattern was observed. These increases in the number of calls are attributed to the wet season experience in the state of Kelantan.

By looking at the total number of ambulance calls per capita within a 2-year period of study, it was noted that the district of Kota Bharu, Kelantan with a population of 266,000 had a ratio of 1:155 for the year 2001 and 1:89 in the year 2002 (15-18). Taking all these information together the results obtained confirmed that the district of Kota Bharu is relatively not a busy area in terms of the number of emergency cases. In the absent of any published data in other areas in Malaysia, a comparison can only be done with that of internationally established data. For example is Canada, the Emergency Medical Services of Manitoba (2002) reported that some Regional Health Authority (RHA) in that particular area received a significant number of ambulance calls per-capita in the year 2002. These RHA are Assiniboine, which recorded a ratio of 1: 13 from a total population of 71,497 people, Brondon had 1: 12 (populations 47,652), Burntwood recorded a ratio of 1: 7 from populations of 44, 806 and lastly, Winnipeg showed a ratio of 1: 1.2 (populations of 65,728) (19). Smith (2001) in his study found that rural area in Australia with population of 413,026 people managed to receive a total of 20,000 ambulance calls for the years of 1996 and 1997 (20). Thus the ratio of number of calls to population of 1: 20.7 seem to indicate a possible norm. As such the figures for district of Kota Bharu is low as compared to that of the international records ratios.

This observation may be related to the absent of proper method of data recording for ambulance calls, lack of interest in calling for the ambulance services amongst the local population or perhaps the sheer lack of facilities to make the call. On the other hand it is also possible that data of ambulance calls and other information may be missing or left unrecorded. Our inquiries to the ambulance services at HUSM and HKB confirmed that differences in the recording system for telephony at these places could further complicate and may contribute to the lack of documentation.

This situation correlates well with the findings of this study where it was found that there is no standard method of data recording for both the hospitals. Altintas & Bilir (2001) in their study also faced with a similar problem in Turkey (10). These authors mentioned that due to financial problems the ambulance crew and dispatchers of Ankara

EARS recorded their times manually. This observation is further emphasized by another study in Japan where it was concluded that if digital and electronics recording systems were utilized time recordings can be more precise (21). The situation may be the same for HUSM and HKB in the Kota Bharu District.

Emergency ambulance services are present at the both HUSM and HKB. However, as mentioned earlier, the quality of pre-hospital care services, especially emergency ambulance services is found neglecting due the possible lack of funds allocated for the development of vital components within this service. It is also possible that the lack of proper planning during the initial phase of the development of these hospitals could have contributed to this state of affair. Budgeting for these areas may be categorized as of low priority. Further, there were no single body or organization that has taken serious attention in this matter. This situation could be rectified by establishing an independent body to oversee all the Emergency services under one body as can be seen in most European countries and North America. In these countries, EMS is totally run by dedicated single body or organization.

In the USA for example, they have a special body to take care of EMS system including ambulance services. Two studies stated that in the state of California, a special organization called The California Emergency Medical Services Authority (CEMSA) play the crucial part in managing Emergency Medical Services (EMS) in that state (22, 23). This Authority also trains ambulance personnel up to level of Emergency Medical Technician (EMT). They also develop protocols in the management pre-hospital care and prepare career pathways for their staffs, which include courses and programmed leading to bachelors and advanced degrees. Whereas in Malaysia, the EMS is totally run by the same personal who are also hospital staffs. The drivers, medical assistants and staff nurses are all under the hospital payroll and at the same time given task to manage EMS. This system is seemed to be difficult to run properly. It is suggested, therefore, that there should be a dedicated body with their own staffs managing the EMS system, which is thought to be the best way to preserve better outcomes (24).

The present study pre-empt that ambulances services in the local setting may require modifications and adjustments if not a major overhaul. The philosophy and function of EMS system in Malaysia is still unclear and substantial attentions from the authorities are needed. No studies have been reported with regards to the efficiency of neither the emergency services nor the ambulance response time at these two hospitals under study. In fact no similar studies have been done for the country. In the absence of formal study to determine either efficiency or the extended services of the EMS system in Kota Bharu region, it is reasonable; therefore, to conclude that this paper may provide the initial impetus towards a more detailed and comprehensive analysis of the EMS in Malaysia.

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ORIGINAL ARTICLE

IMPACT OF A SPREADING EPIDEMIC ON MEDICAL STUDENTS

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The emergence of severe acute respiratory syndrome (SARS) had caused fear and anxiety of unprecedented proportion. To examine the impact of SARS on the medical students in a private medical university, a self-reporting questionnaire study was carried out to assess the factual knowledge, anxiety level and perception of the crisis, among the students. The two-week study (between 12 and 23 May, 2003) was carried out three weeks after the first reported SARS-related death in Malaysia. Ninety-one Phase I (junior) and 113 Phase II (senior) students completed the questionnaires. A large majority of students of Phase I and II were correct in their factual knowledge and were sensible in their perception of the future and the handling of the crisis by government(s). However, phase 1 students expressed significantly greater degree of anxiety compared to Phase II in relation to attendance and personal protection in hospital, and in meeting people coughing in public places. The lesser degree of anxiety expressed by phase II senior students may be due in part, to a more realistic assessment of SARS risk brought about by maturity, time spent in hospital and interaction with clinical lecturers and medical staff.

***Key words**: severe acute respiratory syndrome, SARS, medical students, knowledge, anxiety, perception, Malaysia*

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Introduction

On the 15th March 2003, the World Health Organization issued a rare emergency travel advisory as a global alert to a readily transmissible new respiratory disease, named Severe Acute Respiratory Syndrome or SARS. It showed clear capacity for spread along the routes of international air travel and in densely populated areas. Countries and cities especially Hong Kong, Beijing, Toronto, Singapore and Taiwan had suffered severe socio-psychological and economic consequences from the rampage of SARS (1). Although there had not been any local transmission of SARS in Malaysia, its close travel ties with the surrounding countries and cities affected by SARS, in particular Singapore, had caused much concern and anxiety among the Malaysian public and healthcare workers.

During the crisis, the medical students' teachings in hospitals in Hong Kong (2), Toronto (3) were completed halted because of SARS epidemic occurring in these sites. In our private medical university, while hospital teaching for students continued, there was a heightened emphasis for stringent infection control measures such as meticulous hand washing, appropriate use of respiratory mask, and banned entry to designated SARS isolation wards. We also banned all teachings in Accident & Emergency Departments of our three teaching hospitals and the entry points of university-affiliated health clinics where the screening of suspected SARS patients occurred. Furthermore, all student electives to all SARS-affected areas were prohibited. One of the authors (LCL) of this paper chaired a medical advisory panel that was established to make recommendations to the university on SARS-related policies for staff and students. The author conducted an urgent three-day compulsory briefing for all students (Phase I and II) during the start of the SARS epidemic in March 2003, on the precautionary steps against SARS and the university policies of ensuring student safety.

To investigate the impact of the SARS crisis on our medical students, a questionnaire study was conducted among the undergraduate students over a two-week period (between 12 and 23 May, 2003),

Table 1 : Background of medical students according to Phase 1 and II

	N recorded	Entire Group	Phase		p
			One	Two	
Male %	204	40.7	42.9	38.9	0.571
Mean age (SD), yrs	204	2 1(1.3)	21 (0.9)	23 (1.0)	<0.001
Primary source of information					
Newspaper	204	63.2	68.1	59.3	-
Internet	204	9.8	6.6	12.4	-
TV	204	5.4	5.5	5.3	-
Conversation with people	204	21.6	19.8	23.0	0.458
If newspaper is the primary source, which newspaper?					
Malay	112	0.8	0	1.5	-
English	112	70.5	62.5	77.3	-
Chinese	112	22.1	32.1	13.6	-
English & Chinese	112	5.7	5.4	6.1	-
English & Malay	112	0.8	0	1.5	0.119

initiated three weeks after Malaysia reported its first SARS-related death (accumulative figures at study commencement were two SARS-related death and six probable SARS cases). While there was no evidence of local transmission in Malaysia then, other areas such as China, Hong Kong and Singapore were facing a hard time to contain local transmission of SARS disease (4).

The objective of our study were twofold: first, to examine the factual knowledge, anxiety level and perception of students on the current SARS crisis; second, to compare between the Phase I and Phase II students, their factual knowledge (based on the current understanding of SARS then), anxiety level and the perception of the future for SARS and the handling by the international and Malaysian governments.

In our curriculum, students are exposed to hospital ward teaching from first year and the amount of hospital ward teaching increases exponentially from the second half of the third year when they enter Phase II. On average, Phase I students spent about half a day in one or two weeks in hospital ward teaching, interacting with real patients. Phase II however would spend almost everyday in hospital or health clinics with real patients as the requirement for Phase II curriculum. We tested the hypothesis that Phase II students, compared to Phase I, had better understanding of SARS but were more anxious about the risk of exposure to SARS because of their time spent in hospital wards. We also hypothesized that Phase II students had a more realistic perception on the future for the crisis and about the actions taken by the international and Malaysian governments.

Subjects & Methods

Two hundred and twenty medical students [consisting of the Phase I Semester 3 & 5 students (n=100) and Phase II Semester 7 & 9 students (n=120)] of the International Medical University, Malaysia, were invited to participate in a self-reporting questionnaire study. The rationale for choosing these groups was because they had completed one year into their respective phases. The two-page questionnaire were completed anonymously and consisted of closed questions relating to students' background, their level of factual knowledge on SARS (based on the understanding of the disease at that time), anxiety level during hospital attendance and in public places, and their personal perception on the SARS future and the handling by the international and Malaysian governments. Respondents answered from categories of answers, some of which requiring a 4-point scale (e.g. questions related to anxiety level and perception of severity). A pilot study of the questionnaires was carried out with 5 medical students and 3 doctors and amendments made wherever necessary. Incorrectly filled questionnaires (n=16) were excluded from analysis (*e.g.* two answers were given when only one answer was asked for). Differences in results between the Phase

Table 2 : Factual knowledge on SARS according to Phase 1 and 2 medical students

	N recorded	Entire Group	Phase		p
			One	Two	
Primary microbial agent					
Corona virus	202	96.5	97.8	95.6	-
Paramyxovirus	202	2.5	1.1	3.5	-
Respiratory Syncytial Virus	202	2.0	1.1	0.9	0.541
Primary mode of transmission					
Via infected aerosol droplets	200	81.5	87.6	76.6	-
Airborne	200	18.0	12.4	22.5	-
Sexual transmission	200	0.5	0	0.9	-
Contaminated food	200	0	0	0	0.112
Best-recognised clinical presentation					
High fever>38°C, SOB, cough, flu-like	204	99.5	100.0	99.1	-
Headache, tinnitus, abdominal pain	204	0.5	0	0.9	-
Cough, joint pain, skin rash	204	0	0	0	-
Lethargy, weight loss, anorexia	204	0	0	0	0.368
Recommended 'standard' treatment					
No specific treatment except for supportive	201	52.7	46.1	58.0	-
Anti-viral drugs +/- steroids	201	43.8	48.3	40.2	-
Palliative treatment	201	3.5	5.6	1.8	-
Pneumectomy	201	0	0	0	0.123
Countries with high local transmission					
Hong Kong & Mainland China	204	99.0	100.0	98.2	-
Malaysia	204	0.5	0	0.9	-
Korea	204	0.5	0	0.9	-
India	204	0	0	0	0.443
Effective individual protection against virus transmission EXCEPT					
Eating imported canned food from China	203	96.1	98.9	93.8	-
Constant hand washing & personal hygiene	203	2.5	1.1	3.6	-
Wearing of protective mask	203	1.5	0	2.7	-
Avoid overcrowded places	203	0	0	0	0.148

I and II students were assessed using two-tailed Chi Square tests (or two-tailed Fisher exact tests when the expected cell frequency was less than five). A $p < 0.05$ was considered as significant. The analysis was performed with statistical software, SPSS' Version 11.0 for Windows.

Results

Two hundred and four students [91 Phase I (60.6%) students and 104 (69.3%) Phase II students] completed the questionnaire satisfactorily. Of the respondents, nearly 60% was female. The gender differences between the two groups (Phase 1 and II) were comparable. Mean age in Phase II was significantly higher than in Phase I. Most derived their information from newspaper (63%), followed by conversation with people (21%), internet (9%) and TV (5%). Of the 112 students who selected newspaper as their primary source of information, 70% read English newspaper and 22% used Chinese newspaper. The proportions were comparable between the two groups (Table 1).

Almost all (96%) quoted *Coronovirus* as the

Table 3 : Anxiety levels according to phase 1 and II medical students

	Total	Phase I	Phase II	p
Going into hospital wards	(n=201)			
Comfortable and not anxious because of hospital precautions put in place	36.8	18.2	51.3	-
Anxious but comfortable	40.3	36.4	43.4	-
Anxious but uncomfortable	16.4	33.0	3.5	-
Frightened to go	6.5	12.5	1.8	<0.001
Personal protection while in open wards	(n=202)			
Confident without any form of protection but frequent hand washing is required	47.0	35.6	56.3	-
Wear mask only in certain circumstances	38.1	35.6	40.2	-
Wear mask all the time	10.9	22.2	1.8	-
Need full protection gear to feel safe	4.0	6.7	1.8	<0.001
Meeting people actively coughing in public places	(n=203)			
I don't mind and carry on as usual	19.2	8.9	27.4	-
Avoid him because of unknown cause for his cough	71.9	78.9	66.4	-
Avoid him immediately because I suspect he has SARS	5.4	6.7	4.4	-
I will insist on the person be screened in the hospital and quarantined	3.4	5.6	1.8	0.006

primary microbial agent implicated in SARS. There was one student from each group who wrongly implicated Respiratory Syncytial Virus. 81% correctly reported infected aerosol droplets as the primary mode of transmission while 18% reported 'airborne'. One student in Phase II was completely wrong by reporting 'sexual transmission' as the primary route of transmission. Almost all (99%) correctly reported high fever ≥ 38∞C, breathlessness, cough and flu-like symptoms, as the 'best-recognised' clinical presentation. Most correctly identified 'no specific treatment except for supportive' (52%) and 'anti-viral drug +/- steroids' (43%) as the recommended standard treatment. For countries with high local transmission, almost all (99%) correctly stated Hong Kong & Mainland China. In Phase II group, one wrongly stated Malaysia and another one stated Korea. On the *exception* to effective individual protection against transmission listed, most (96%) corrected selected 'eating imported canned food from China'. However, one student from Phase I and four students from Phase II selected 'constant hand washing and personal hygiene' as their answers, while another three in Phase II selected 'wearing of protective mask'. The selection of wrong answers for these students was likely to be due to misinterpretation of this question where the exception was asked for. In all these questions, the answers were comparable in both groups of students (Table 2).

Questions concerning going into the hospital wards revealed that 40% were anxious but comfortable about this, while 36% were comfortable and not anxious due to the hospital precautions put in place. 16% were anxious and uncomfortable about going while 6% were frightened to go. There was a significant difference between the two groups in that over 90% of the Phase II students were comfortable about this, compared to just over 50% of the phase I students, and about 45% of Phase I students were either uncomfortable or even frightened to go into hospital, compared to only 5% in Phase II (Table 3).

Regarding personal protection in open wards, 47% were confident without any form of protection but would exercise frequent hand washing, and 38% would wear mask only in certain circumstances. 10% would wear mask at all times while 4% would need full protection gear in order to feel safe. The pattern of answering was significantly different between the two groups in that over 96% of Phase II students were confident without any form of protection or wear mask only in certain circumstances, compared to about 72% in Phase I. Similarly, about 3% of Phase II students would wear mask at all times or require full protection gear in order to feel safe, compared to almost 30% in Phase I (Table 3).

When meeting people in public places who actively cough, most (71%) would avoid him because of the unknown cause for his cough. 19%

Table 4 : Medical students' perception of the crisis and its handling according to Phase 1 and II

	Total	Phase 1	Phase II	p
My perception on the crisis	(n=202)			0.505
Will terminate within weeks	3.5	2.2	4.4	
Number has now peaked and will subside in the next few months	44.6	42.7	46.0	
Live with us in stable state, neither worsening or getting better	47.0	51.7	43.4	
Deteriote with millions dying within the next few years	5.0	3.4	6.2	
Information by Malaysian Government	(n=203)			0.152
Too much has been given	1.5	0	2.7	
Adequate and transparent	27.1	22.2	31.0	
Still attempts to hold back information	60.6	67.8	54.9	
Top level conspiracy to conceal the real severity	10.8	10.0	11.5	
On existing international and Malaysian measures to control SARS spread	(n=204)			0.727
Too tight and many	0.5	0	0.9	
Sufficient	43.1	40.7	45.1	
Insufficient	44.1	46.2	42.5	
Still severely lacking	12.3	13.2	11.5	

stated that they would not mind and would carry on as usual, while some would avoid him immediately because of suspicion of SARS (5%) or insist on the person be screened in the hospital and quarantined (3%). There was significant difference between the two groups in that compared to Phase I, proportionately more students in Phase II would either carry on as usual or avoid active coughers because of unknown cause of his cough, and proportionately fewer in Phase II would suspect that active coughers had SARS or insisted on screening and quarantined in hospital (Table 3).

With regards to perception on the future of the crisis, 47% stated that SARS would live in human population in stable state, neither worsening or better, while 44% stated that the number had now peaked and would subside in the next few months. Small proportions stated that the crisis would terminate within weeks (3%) or deteriorates with millions of people dying within the next few years (5%). The pattern was similar and comparable in both groups of students (Table IV).

More than half the students (60%) believed that there had been attempts by the Malaysian government to hold back information, while about a quarter (27%) reckoned that the government had been transparent and their information adequate. Around 10% believed that there was a top-level government conspiracy to conceal the true degree of severity while very few (1%) felt that too much information had been provided to the general public. The pattern was similar in both groups of students (Table 4). With regards to the measures taken by international and Malaysian governments to control SARS, almost similar proportions of students reckoned that they were sufficient (43%) and insufficient (44%). 12% of the students perceived that the measures were severely lacking, while only one student from the Phase II group reckoned that they were too tight and many. Overall, the two groups were comparable in these (Table 4).

Discussion

We have shown that while a large majority of students in both groups was correct in their factual knowledge and sensible in their perception of SARS crisis, Phase I students compared to Phase II, expressed greater degree of anxiety about being in hospital, adequacy of individual protection against SARS and meeting people who cough in public places. A small but comparable proportion of students in both groups were pessimistic about the future outlook on SARS and some 10%, from both groups, were highly suspicious and critical of governments' dealing with public information and the control measures.

Fear and anxiety caused by spreading epidemics of known or unknown diseases can have damaging consequences on people and economy.

Never before in the era of modern world with its advances in medicine has a disease like SARS that had caused a psychological impact of such global scale (1, 5-6). At the stage of the study, the public and healthcare worry was at its peak with countries like Hong Kong, Singapore and parts of China struggling to contain the escalating number of local cases. Our key teaching hospital, where both phases of students attended for ward teaching, was a designated SARS hospital for Malaysia where suspected cases were sent in for screening and isolation. As such, students and parents alike were understandably concerned of the possible risk of contracting SARS and about the adequacy of hospital screening and control measures.

Contrary to our hypothesis, both phase I and II students showed comparable level in their understanding on SARS, and perceived similarly on SARS future and its handling by governments. Surprisingly however, phase I students, compared to phase II, were much more anxious about being in hospital and about the crisis. This discrepancy in their level of anxiety could not be explained by their source of information or the types of newspapers they read since these variables were not significantly different in both groups.

One plausible explanation is that Phase I students, compared to Phase II, exhibited greater degree of health anxiety. Health anxiety refers to the phenomena of medical students having excessive anxiety about their health that had often lead to frequent request for medical consultations and sometimes, needless investigations. It has been termed variously as medical student's disease, nosophobia and medicalstudentitis (7-8). While the existence of such a condition in medical students is still debatable (9), there is no evidence to show that junior students experience this to a greater degree than their senior counterparts, or even a suggestion of this.

Another explanation may be simply the fact that Phase II students, by having more contacts with the hospital, clinical lecturers and medical staff, had a more realistic assessment of the SARS risk. They might have also observed first hand the stringent measures put in place for the screening and isolation of suspected SARS patients. This in turn helped to build confidence and provide reassurance that the risk of contracting SARS was minimal. Finally, the chronological maturity of Phase II students in medical knowledge and as an individual, compared to Phase I might play some role in avoiding excessive anxiety in these circumstances. In one study on Hong Kong medical and nursing students (10), it is worth noting that when compared to non-healthcare students, they exhibited a significantly greater degree of anxiety and psychological stress.

In general, most students of both the phases held realistic view on the future for SARS in that the answers selected were a reflection of the feelings among the experts at that time. A majority of students from both phases appeared skeptical at the government information on the statistics of cases presented to the general public, and some 10% from both groups would even believe that there had been top-level conspiracy to conceal the true severity. This is perhaps understandable, considering what previously had taken place in Malaysia where some degree of deliberate concealing of information by the government was deemed necessary. The students appeared to be 'divided' in their perception of the adequacy of infection control measures put in by WHO and Malaysia. Looking back now, it is obvious that they had worked, supporting the notion that they were adequate at the time.

Our novel findings from this study implies that there is a need to address the anxiety status among the medical students that goes beyond the imparting of factual knowledge. The training needs between the junior and senior students may require to be probed into separately in the overall effort to improve the training of our future medical doctors. Perhaps, as what was said by Professor Sydney Chung, Dean, Chinese University of Hong Kong during the crisis 'It is precisely at the time of such a major medical crisis that educating the next generation of doctors becomes so important', it may be apt for us to think of ways to use such future crisis as an opportunity to better educate our medical students (2).

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ORIGINAL ARTICLE

USAGE OF TRADITIONAL MEDICINES AMONG ELDERLY AND THE PREVALENCE OF PREDNISOLONE CONTAMINATION

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The elderly consume many medications including traditional medicines. In 1986, it was found that 29% of elderly took traditional medicines although in 1996, the National Health Morbidity survey reported a 2.3% prevalence. However, studies from other countries showed much higher percentages. The Ministry of Health in Malaysia is concerned that some of these preparations maybe contaminated with steroids, antihistamines, hormones and other poisons. The aims of the study were to determine a). the health seeking behaviour of elderly Malays living in rural areas, b). the utilization of both modern and traditional medicines and c). the steroid content of the traditional medicines used. Methodology included interviews using structured questionnaires of elderly Malays living in rural areas of Kelantan, aged above 60 years. Samples of traditional medications collected were sent to the Pharmacology Department, School of Medical Sciences, Universiti Sains Malaysia, for steroid content analysis using Thin Layer Chromatography. A total of 599 elderly respondents were interviewed comprising 62.4% females and 37.6% males. The 60-69 years cohort group made up 48.7%, followed by 70-79 years at 36.1% and the remainder 15.2% were more than 80 years. There were 82% of elderly taking medicines. The trends of utilization of modern and traditional medicine in the last two weeks among elderly were 59.3% and 40.9% respectively. The utilization of traditional medicine by rural elderly Malays was therefore much higher than that reported in the previous study and nearly similar to that of France and Australian studies. There were 102 samples of traditional medications collected and analysed for steroid content. Results showed that 27.5% were positive for prednisolone, 34.3% positive for unknown steroids (a total of 61.8%) and 38.2% were negative for both steroids. The present study therefore once again confirmed the high usage of traditional medicines where some of which are contaminated with steroids.

Key words *: Rural elderly, traditional medicines, steroids, prednisolone*

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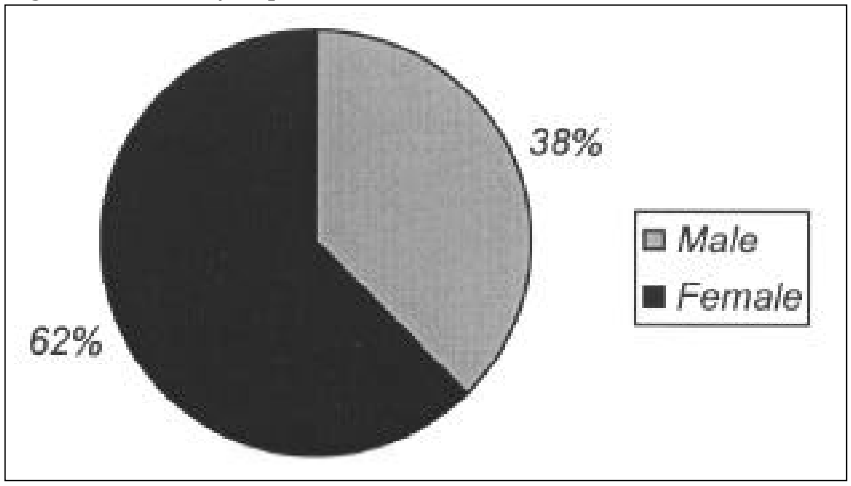
Introduction

Aging is usually seen as a progressive, generalized impairment of functions resulting in a loss of adaptive response to stress and in growing risk of age associated disease (1). In Malaysia, the population of elderly is increasing due to an increase in life expectancy, and improved health care services. Elderly is defined as those aged 60 years and above, as recommended by Malaysian Medical Association (MMA). Data from Lembaga Penduduk dan Pembangunan Keluarga Negara (LPKKN) estimated that the number of elderly, which exceeded 65 years of age will increase from 3.9% in the year 2000 to 6.1% by the year 2020 (2). The 2nd National Health Morbidity survey in the year 1996 showed that

Figure 1: Sex of respondents



females lived longer than males and that the ratio between them was two females for every one male (4). In 1999, the life expectancy at birth for males in Malaysia was 69.6 years; meanwhile for females it was 74.6 years (3). This disparity in life expectancy is expected to result in further demographic changes whereby the female elderly population will increase further and thus feminization the elderly population.

A study was sponsored by the World Health Organization (WHO) on the Topic of "Health and Aging in Malaysia" in 1986 (5) with the aim to assist the authorities in identifying health and social problems pertaining to the elderly. It focused on demographic profile of the aged, examined the health and functional ability, mental health, uses of health services, living conditions and social participation of the elderly in Malaysia. The study found that 29% of the elderly took traditional medications, which were either Malay or Chinese herbal medicines (5).

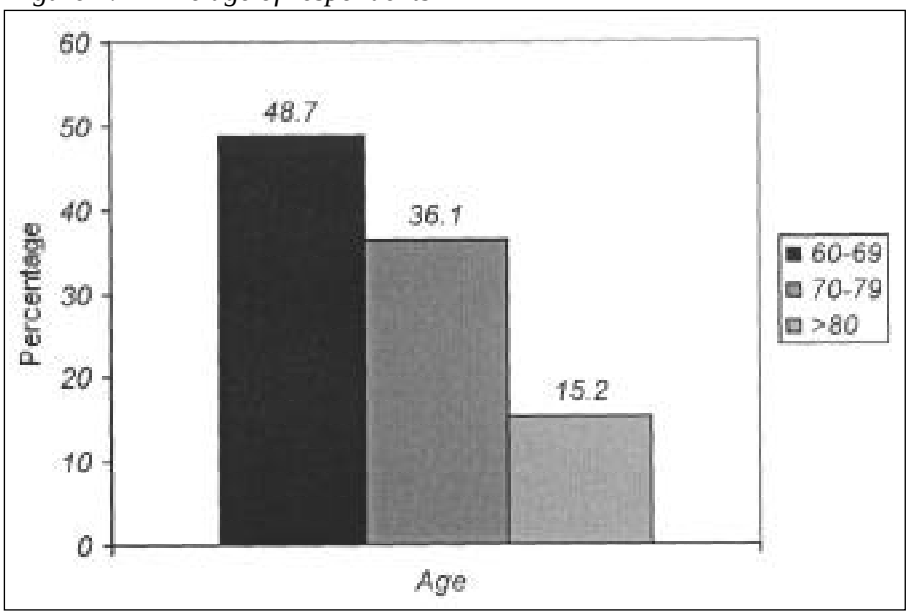
Ten years later in 1996 the National Health Morbidity survey reported that 2.3% of the elderly population utilized traditional medicines during a two week recall (4). However, data from other countries have shown a much higher percentage of the population using traditional medications, e.g. 49% in France, 33% in USA, 24% in Denmark, 60% in Hong Kong and 48.5% in Australia admitted to using traditional medications. It is assumed that the utilization of traditional medicine in Malaysia is much higher than that reported in the previous study. Apart from the widespread usage of traditional medicines, there is a constant worry that some of traditional medicines available in the market may contain contaminants such as steroids, local anaesthetics, hormones and other poisons.

In developing countries like Malaysia, there is a lack of attention given to issues and problems related to the elderly. However, the stakeholders in health care such as the government, private doctors, pharmacists and researchers should be aware of the issues faced by elderly people (6). The utilization of traditional medicines is still questionable, and among experts, questions arise regarding the quality, efficacy, contents and safety of its usage. There are many unregistered traditional medicines sold widely in this country. At the same time, the problem becomes more serious because the society is using traditional medicines consisting of either those that are produced locally or those that are imported.

Traditional medicine is defined as non-prescription drugs bought over the counter or by direct selling. It contains herbal/natural products, which are consumed orally either as powder, tablet, capsule, caplet, emulsion, suspension, mixtures or boiled preparations. These include Pharmaton, Zinaxin, Remifemin or other herbal containining products. On the other hand, modern medicines are defined as prescription drugs obtained from physicians or over the counter that do not contain herbal/natural ingredients in them.

Ministry of Health is very concerned with the utilization of traditional medicines by consumers in Malaysia because these drugs do not only contain herbs but may also contain contaminants such as steroids, antihistamines, local anaesthetics, hormones and other poisons (7, 8). Some unregistered traditional drugs are contaminated with high contents of heavy metals such as lead, mercury, and arsenic (7). Mercury can cause vomiting, bleeding, diarrhea, disturbance of nervous and renal functions, while lead can cause anaemia and lead to disturbances of nervous and mental functions. Traditional drugs containing dexamethasone can cause swelling of the face, brittle bones and renal failure, as well as delay the healing process from diseases (7).

Figure 2: The age of respondents



Traditional medications that are contaminated with steroids raise a lot of concerns especially if taken over a long-term (9). Chronic use of contaminated traditional medications that contain prednisolone for example, will cause problems. Prednisolone is one of the steroids that can reduce swelling and decrease the body's immune response (10). However, long-term usage will weaken the body's immune response and reduce the ability to fight infection, increase blood pressure, bruising, acne, swollen hand, sore or weak muscles. Other side effects include insomnia, nausea, vomiting or stomach upset, muscle weakness or joint pain, increased hair growth and osteoporosis.

The aims of the study therefore were to determine a). the health seeking behaviour of elderly Malays living in rural areas, b). the utilization of both modern and traditional medicines and c). the steroid content of the traditional medicines used.

Methodology

The study was conducted from December 2000 to December 2002, which involved elderly Malays aged above 60 years and living in rural areas of Kelantan. The study was approved by the Research and Ethics Committee of the School of Medical Sciences, Universiti Sains Malaysia.

Based on previous studies, and taking the prevalence rate of usage of traditional medicine of 30% and the margin of error at 5%, a minimum sample size of 323 participants was calculated. However, in this study a sample size of 599 people was used so that the margin of error can be reduced to 4%.

Medical students in years two and three, who were involved in "Community Family Case Studies" in Wakaf Bharu and Tumpat areas together with the help of research officers conducted the interviews. With the cooperation of Penggawa and the use of voting list, only houses with elderly were chosen for the study.

Pretesting of the questionnaire was done using 40 elderly participants in Kampung Laut, Tumpat and the results were encouraging.

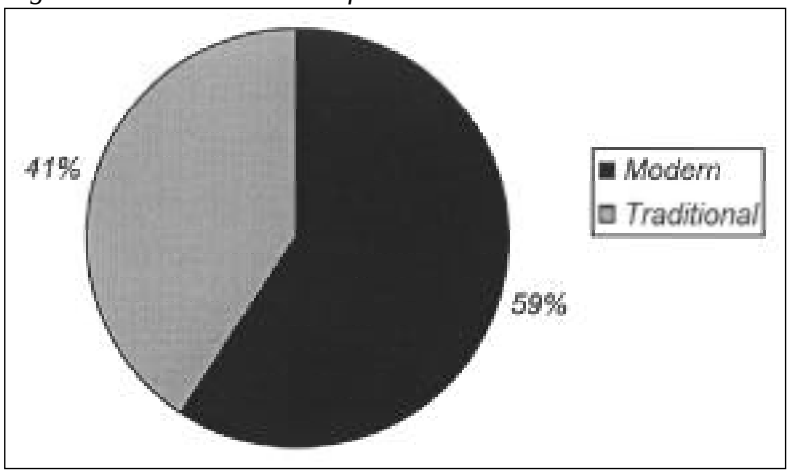
The elderly were interviewed by either a medical student or a research officer and the data were collected using a structured questionnaire (Appendix A). The questionnaire consisted of two sections. Section A included a personal profile and socio-demographic data (name, age, sex etc). Meanwhile section B contained questions pertaining to their health-seeking behaviour during the last 2 weeks and record of medications taken during the last two weeks (types of drug, source and name of drug etc).

Those who took traditional medicines were requested to hand over the said medications and were compensated for their time and cost of traditional medicines. All samples of traditional medicines were labeled accordingly and kept in the filing cabinets for analysis of steroids.

Analysis of steroid.

Samples of traditional medicines collected from the elderly were tested for steroid content using Thin Layer Chromatography (TLC), which was based on a multistage distribution process (11, 12).

Figure 3: The utilization of modern and traditional medicine



The process involved a suitable adsorbent (stationary phase), solvent or solvent mixtures (mobile phase or eluent) and the sample molecule, in this case the steroid. Prednisolone was used as a standard. The adsorbent of TLC was coated as a thin layer onto a suitable support (e.g. glass plate or polyester or aluminium sheet). The substances were separated by elution with a suitable solvent on this layer.

Traditional medicine samples were initially pretreated by mechanical crushing, extraction, filtration or cleaning up to remove undesired impurities. The extracted product was then spotted on a TLC plate. In our laboratory we use aluminium sheet with silica layer of 0.25 mm in thickness and the particle size of silica was between 5 to 17 mm.

The chromatograms were developed by putting the plates into a glass TLC development tank with mobile phase consisting of chloroform: methanol: water at a ratio of 64:50:10 respectively. The mobile phase was prepared daily because its composition changes due to chemical reaction and evaporation.

Chromatograms were normally developed to 10 cm from the origin and left to dry. The developed and dried TLC plates were then sprayed with reagents for easy spotting of the compound. The reagent used was 5% sulphuric acid in ethanol, followed by 1% vanillin in ethanol. TLC plates were left to dry in the oven at a temperature of 110^{o} C for 5 to 10 minutes. Using UV scanner the spot was identified as blue or yellow for prednisolone and that of standard.

Results

A total of 599 elderly respondents were personally interviewed by the medical students or research officers in the villages of Wakaf Baru and Tumpat in Kelantan. The elderly respondents interviewed consisted of 62.4% females and 37.6% males (Figure 1).

The elderly were subdivided into three age groups, i.e. 60-69, 70-79 and more than 80 years. The respondents aged 60-69 years made up the highest percentage of respondents with 48.7%, followed by 36.1% aged 70-79 years and 15.2% were above 80 years of age (Figure 2).

a. Health seeking behaviour

Majority of the elderly (82%) were currently taking many different types of medications, including modern medicines obtained from the hospitals, clinics or pharmacy and or traditional medicines obtained from shops, friends, pharmacy or prepared personally.

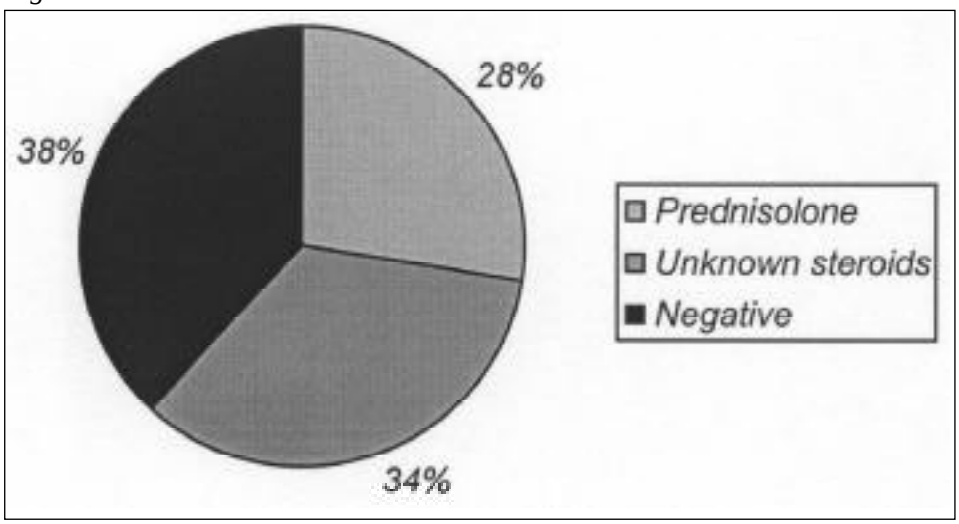
b. Utilization of modern and traditional medicines

The trends of utilization of medicines in the last two weeks among elderly Malays in the villages studied in Kelantan showed that 59.3% took modern and 40.9% took traditional medicines (Figure 3).

For the 60-69-year old group, 28.4% took modern and 22.5% took traditional medicines. Meanwhile, the elderly aged above 70 and 80 years, considered as middle and late elderly respectively, took 30.9% modern and 18.4% of traditional medicines respectively.

However, there were 18% of elderly who did not consume any type of medicine. Overall, the present study showed that the utilization of traditional medicine among elderly Malays aged, between 60 and 90 years was 40.9%.

Figure 4: The steroid contents



c. Steroid content of traditional medicines

There were 102 traditional medicine samples collected from the participants. These traditional medicines were willingly given to the researchers and kept in filing cabinets or mini refrigerator for analysis at a later date. The samples came in the form of powder, tablets, capsules, emulsion, suspension or mixtures. Samples were analysed for steroids using TLC, with 5 samples at a time to save cost and time. The results showed that 27.5% of the collected traditional medications were positive for prednisolone, a standard that is available in the Pharmacology Department, 34.3% were positive for unknown steroids and 38.2% were negative for both steroids (Figure 4). Thus a large number of samples collected (61.8%) contained steroids.

Discussion

Most respondents were not able to read and write, thus most of the questionnaires were filled up by the interviewers. The result of the survey showed that the proportion of females in the sample was higher when compared to the males (Fig 1).

This is somewhat expected as life expectancy of females is somewhat higher than males i.e. 69.6 years for males and 74.6 years for females (3), which would naturally result in a larger female group in a population of that age range. Majority of elderly surveyed took medications. Physiological changes taking place in the elderly will affect the pharmacology of medications taken especially the pharmacokinetics and pharmacodynamics (14). Polypharmacy iscommon in the elderly (15).

This percentage of 82% of elderly taking different medications is high and it showed that drugs were an important aspect of their life. The study revealed that modern medicine is still the drug of choice among elderly to cure their diseases compared to traditional medicine (Fig 3). Thus overall, our community still believed in modern medicines to prevent their diseases than traditional medicines.

The modernization and efficient health care system in Malaysia, such as hospital facilities and rural health centres, encouraged them to seek treatment at these facilities and consume prescribed medicines. However, there were 18% of elderly who did not consume any types of medicines. The reason might be that they do not have any diseases during the last 2 weeks recall or might be seeking other non-drug alternative treatments such as homeopathy, wave treatment, colour therapy, vibrotherapy and aromatherapy.

The result showed that this percentage of participants (41%) using traditional medicines was much higher than previous two studies in the Malaysian population (4, 5) but was somewhat similar to the percentages reported in France (49%) and Australia (48.5%). The traditional medication was an alternative treatment to the elderly Malays to cure their diseases.

Some of them utilized traditional medicine because of the Malay cultural beliefs that traditional medicine is more effective and do not harm their bodies as they were obtained from plants and thus contained natural materials. The traditional medicines are cheaper and easily available in every shop and through friends in their areas. Also, some of the elderly may be attracted by the persuasion of the sellers who claim that their drugs had a miracle power to cure diseases.

Steroid analysis of the medications revealed that 27.5% were positive for prednisolone while

34.3% were positive for unknown steroids (Fig 4). The results showed that 3 out of 10 traditional medicine samples collected from the elderly in Wakaf Baru and Tumpat, Kelantan contained prednisolone. Prednisolone was used as a control (standard steroid) in this steroid assay. Prednisolone has many side effects, which are detrimental to the health of the elderly.

The precise nature of the other unknown steroids detected in the analyzed traditional medicines is unclear but may consist of dexamethasone or natural steroids, which exist in plants.

Conclusion

Our study concluded that the health seeking behaviour of the elderly living in rural areas of Kelantan is high. The elderly studied used more modern medicines in comparison to traditional medicines. Overall, the present study showed that the utilization of traditional medicine was 40.9% in this age group. The study also found that some traditional medicines contained prednisolone and other unknown steroids. This study therefore also confirms reports that some traditional medicines are contaminated with steroids, which are harmful to elderly who consumed them for long term.

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CASE REPORT

THE CO-EXISTENCE OF PURE RED CELL APLASIA AND AUTOIMMUNE HAEMOLYTIC ANAEMIA IN A CHILD WITH MALIGNANT LYMPHOMA

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The association between pure red cell aplasia (PRCA) and autoimmune haemolytic anaemia (AIHA) has rarely been reported. PRCA represents an isolated process, characterized by normochromic, normocytic anaemia, reticulocytopenia and erythroid hypoplasia in the bone marrow, and may be attributable to infection with Parvo virus B19. AIHA is a condition in which peripheral red blood cell destruction is induced by the presence of autoantibodies. However, the co-existence of these conditions is very rare, since only few cases of PRCA and AIHA associated with malignant lymphoma (ML) were reported. A case of PRCA and AIHA was detected and described, for the first time in Malaysia, in a 10-year-old child suffering from non-Hodgkin lymphoma from the Department of Haematology, Universiti Sains Malaysia. Following the induction course of chemotherapy, the patient turned anaemic, with tendency for red cell clumping, reticulocytopenia and anisocytosis. AIHA was suspected in spite of the weak Coomb reaction obtained. The bone marrow aspirate revealed the presence of giant pronormoblasts, suggesting PRCA. Serological tests for Parvo virus and other viruses were negative.

***Key words** : pure red cell aplasia, autoimmune haemolytic anaemia, malignant lymphoma.*

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Introduction

PRCA represents an isolated process, characterized by normochromic normocytic anaemia, reticulocytopenia and erythroid hypoplasia in the bone marrow. It has been attributed to infection with Parvo virus B19 (1,2,3). AIHA is a condition where peripheral red blood cell destruction is induced by the presence of autoantibodies (1). The combinations of these conditions are very rare, since only few cases of PRCA and AIHA associated with malignant lymphoma (ML) have been reported (4,5,6,7). In this report, a rare case of co-existence of pure red cell aplasia (PRCA) and autoimmune haemolytic anaemia (AIHA) in a child suffering from non Hodgkin lymphoma has been detected and described at the Haematology Department, Hospital Universiti Sains Malaysia.

Case Report

The patient was a 10-year old Malay boy who is a known case of non-Hodgkin lymphoma (T-cell type), stage IV, first diagnosed in February 2002, with pulmonary involvement. Histopathological examination of tissue obtained from biopsy of the forearm soft tissue swelling showed features of non Hodgkin lymphoma (T-cell type).

During that time, the full blood picture of the patient showed features of mild anaemia (table 1). The blood film did not contain any blast cells or other abnormal cells. At diagnosis, bone marrow aspirate (BMA) and trephine biopsy were performed for staging purposes and showed a normal marrow with no evidence of infiltration by malignant cells.The patient was started on the EORTC-VHR

Table 1. The blood counts and bone marrow aspirate findings of the patient throughout the period of the illness.

	At diagnosis Feb 2002	After induction July 2002	During consolidation Feb 2003	Post consolidation April 2003
Hb g/dl	9.3	6.4	4.1	11.6
WBC $x10^9/l$	6.9	4.4	1.2	6.01
Platelets $x10^9/l$	432	372	56	262
RBC clumps	-	+	-	-
BMA	Normal	Giant pronormoblasts	-	-

protocol for ML on the 4th of March, 2002. After the completion of the induction course of chemotherapy in July 2002, the patient became more anaemic in spite of the supportive blood transfusions that he was receiving. His blood counts are shown in table I. An important finding was the anaemia and the reticulocytopenia which were very low for the degree of anaemia.

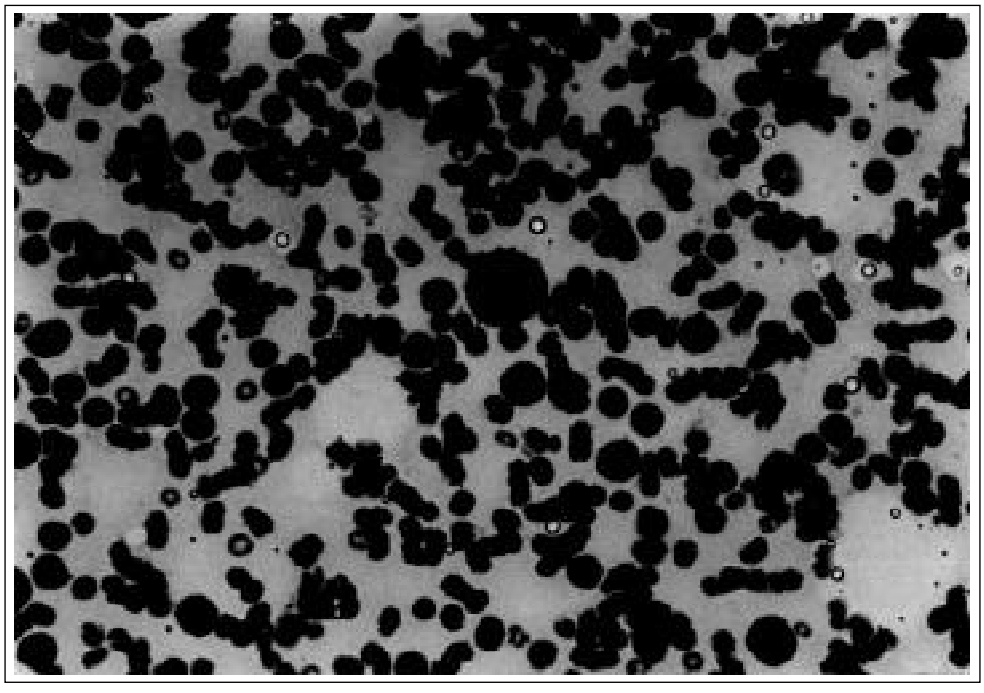
At the same time, the blood film examination revealed red cell clumping, in addition to the lowered red blood cell (RBC) count and anisocytosis. The diagnosis of a cold type AIHA was suspected and a haemolytic work-up was requested. The Direct Coombs test was weakly positive at room temperature, and the serum reactivity showed no definite specificity. The red cell clumping and the weak Coombs reaction both turned negative when tested one week later.

Consequently, another BMA was performed to assess the progress of the disease and to look for a cause for the anaemia. Examination of the BMA smears revealed hypocellularity, with markedly suppressed erythropoiesis, and an estimated 4% of the nucleated elements in the BMA were giant pronormoblasts (plate1). The presence of these giant pronormoblasts is usually associated with Parvo virus infection, and its confirmation requires specific diagnostic serological tests.

Serological investigations for viral infections which included Epstein Barr virus (EBV), Cytomegalovirus (CMV), Hepatitis C virus (HCV), Hepatitis A virus (HAV), Hepatitis B virus and Parvovirus IgM and IgG were all negative.

The patient was, then, maintained on supportive red cell transfusion for his anaemia.The Hb started to rise gradually reaching around 7 g/dl. Consolidation therapy was then started, and the patient was noted to develop pancytopenia which could be attributed to the chemotherapy. His counts at that time are shown in table I.

Plate 1. A giant pronormoblast in the BMA (arrow).



The patient was maintained on the chemotherapy with the support of blood products. He completed the consolidation therapy on 25/2/03. He was maintained on supportive blood and blood products therapy through out the period of the chemotherapy. His latest blood counts are shown in table 1.

Discussion

Parvovirus B19, is a member of the Erythrovirus genus. It is named so because of its tropism for and selective replication in erythroid progenitor cells. Haematological consequences of B19 infection arise due to direct toxic effects on erythroid progenitors in bone marrow with interruption of erythrocyte production (8, 9, 10).

Immunocompromised children, including those undergoing chemotherapy for malignant disorders, are at particular risk of infection with Parvo virus B19 leading to transient aplastic crises. Persistent B19 infection may develop and that will manifest as PRCA and chronic anaemia (11). However, in such patients, the malignancy may obscure the clinical manifestations of the infection, and the attenuated immune responses may obscure serological detection (11,12,13,14,15).The serological tests for Parvo virus B19 were negative in this patient. However, the diagnosis of PRCA due to Parvo virus B19 infection was adopted on the basis of the presence of giant pronormoblasts since many reports have emphasized the lack of serological findings in patients with cancer, especially children (8,11,12,13).

The method of transmission of parvovirus B19 is through respiratory secretions, though infection can also be transmitted by blood and blood products. Among blood donors, approximately 1:10 000- 1:25 000 units of blood during epidemic seasons contain high titres of B19 (8,9). For diagnosing a Parvo virus B19 infection, BMA should be examined, whenever possible (12). A general reduction or even absence, of erythroid precursors, is usually associated with sparing reduction in other bone marrow lineages. Giant pronormoblasts may be visualized and are highly suggestive of Parvo virus B19 infection.

The diagnosis of Parvo virus B19 infection can be performed serologically, or by nucleic acid hybridization assays (14). Most patients with symptomatic infection would have more than 10^5 B19 virions/ml of serum, detectable by dot blot hybridization assays. In acute infections, B19 DNA is usually present at these levels for only 2-4 days . The detection of B19 DNA in serum samples obtained more than two days after the onset of anaemia suggests a chronic parvovirus B19 infection. The sensitivity level of detection of B19 is greatly increased by use of PCR, but has the risk of giving false positive results due to contamination (8). Ultimately, in this case, the diagnosis of Parvo virus B19 infection was not supplemented by any confirmatory test, and was made solely on the detection of giant pronormoblasts in the BMA.

The cold AIHA which the patient developed in association with the PRCA was detected at the same time when the PRCA was suggested. Nevertheless, the weak direct Coombs test, and the absence of clinical signs and symptoms, in addition to the absence of an active haemolytic process, may all be due to the attenuated humoral immune response, as well as the reduced numbers of RBCs in PRCA (4,5,6).

Conclusions

This is the first case report of an association of PRCA and AIHA in a child treated for ML, in Malaysia. Parvovirus B19 should be considered as part of the differential diagnosis in any patient with anaemia associated with low or absent reticulocytes, especially in immunocompromised patients, even in the absence of positive serological tests.

AIHA can occur in immunocompromised children but with a masked picture and can be transient.

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CASE REPORT

EXTRADURAL SPINAL SCHWANNOMA IN 12 YEAR OLD CHILD : A CASE REPORT

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We report a case of a 12 year old girl who presented with cord compression. Imaging studies demonstrated an extradural spinal tumour in the lower thoracic and upper lumbar levels. Histology confirmed the diagnosis of schwannoma while associated findings suggested the possibility of Neurofibromatosis Type I.

Key words *: Extradural spinal schwannoma, neurofibromatosis 1*

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Introduction

Tumors of the spinal canal and its elements comprise of 5-10% of central nervous tumor in paediatric age group. Common extradural tumors in paediatric population are sarcoma, neuroblastoma, teratoma, ganglioneuroma and lymphoma. Schwannoma are rare in this age group. They usually present as intradural mass lesion and present as a dumb-bell shaped tumor and are rarely confined to the extradural space alone.

Case report

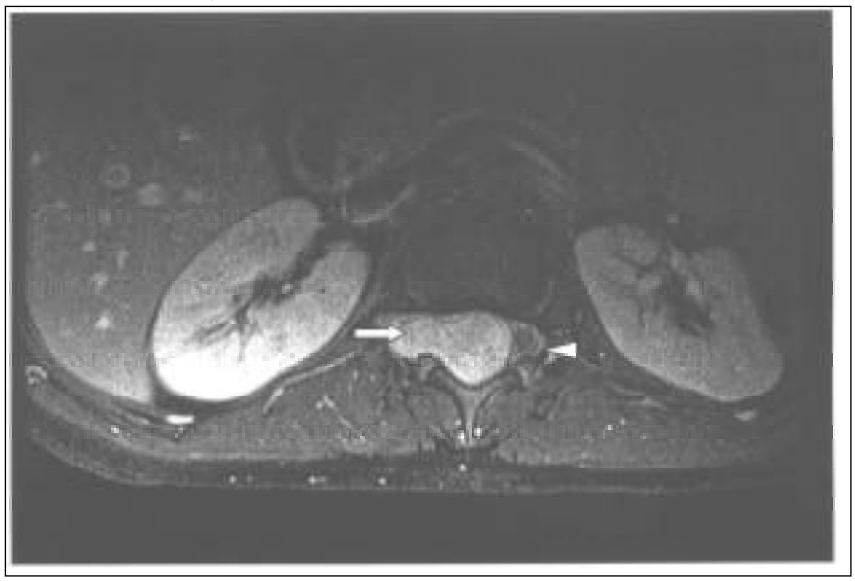
A 12-year-old girl presented with subacute onset of progressive bilateral lower limb weakness. The weakness started from the right lower limb and then affected the left lower limb over a period of 2 weeks. There was presence of radicular pain at T12 dermatome of 2 months duration. She then developed hesitancy of micturation 1 week prior to admission. Past medical history revealed that she had congenital dislocation of left hip which was treated conservatively. The patient was an average student at school with an intelligent quotient (IQ) score of grade IV on Wesler Intelligence for Children.

On examination, there were 4 café au lait spots (ranging from 10-30mm) but no periaxillary/inguinal freckles or subcutaneous neurofibroma. She had horizontal nystagmus, vascular anomalies of both optic discs but with normal visual acuity. Motor system examination revealed no wasting of muscles, mild spascity in both lower limbs, 0/5 muscle power in the left lower limb and 3/5 muscle power in the right lower limb. There were absent knee jerks and brisk ankle jerks with bilateral extensor plantar responses. No sensory level deficit could be detected. There was kyphoscoliosis of the spine. The upper limbs were essentially normal. Sacral reflexes were found to be normal. A formal ophthalmological assessment revealed absence of Lisch nodule.

Routine haemotology and biochemical investigation were normal. Magnetic resonance imaging (MRI) of spine showed an extradural lesion extending from T12 to L1; it was isointense to hypointense in T1 and intensely enhanced with contrast (Fig 1). MRI of brain showed multiple "unidentified bright objects" in the cortex as well as near the ventricles. There was aqueductal stenosis with arrested hydrocephalus but without evidence of raised intracranial pressure.

The patient had underwent surgical excision of tumour via a laminectomy. It was found during surgery that the tumor was well capsulated, firm in consistency and confined only to the extradural space. It extended from T12 to L1. The tumour was totally excised. A diagnosis of schwannoma was

Figure 1: T1 sequence with contrast in axial view showing well circumscribes lesion (arrow) and compressed cord (arrow head).



made on histopathological examination. The tumour was composed of spindle cells with wavy nuclei having hypocellular and hypercellular areas with focal nuclear palisading (Fig.2). The cells stained positive for S100 protein (Fig.3). Post-operatively, patient improved steadily and at 3 months she was able to walk without support. She was able to carry out her activity of daily living independently. Analysis of the NF2 gene was performed using PCR techniques. There was no mutation in this gene.

Discussion

This young girl had findings of café-au-lait spots, aqueductal stenosis with arrested hydrocephalus, mild mental subnormality, kyphoscoliosis, purely extradural schwannoma and UBOs in the certebral cortex. There was no evidence of optic glioma or vestibular schwannoma and family history was negative. These features suggested the diagnosis of neurofibromatosis type 1, although not entirely fulfilling the criteria (1,2).

Figure 2: Schwannoma exhibiting hypercellular (right hand corner) and hypocellular region.

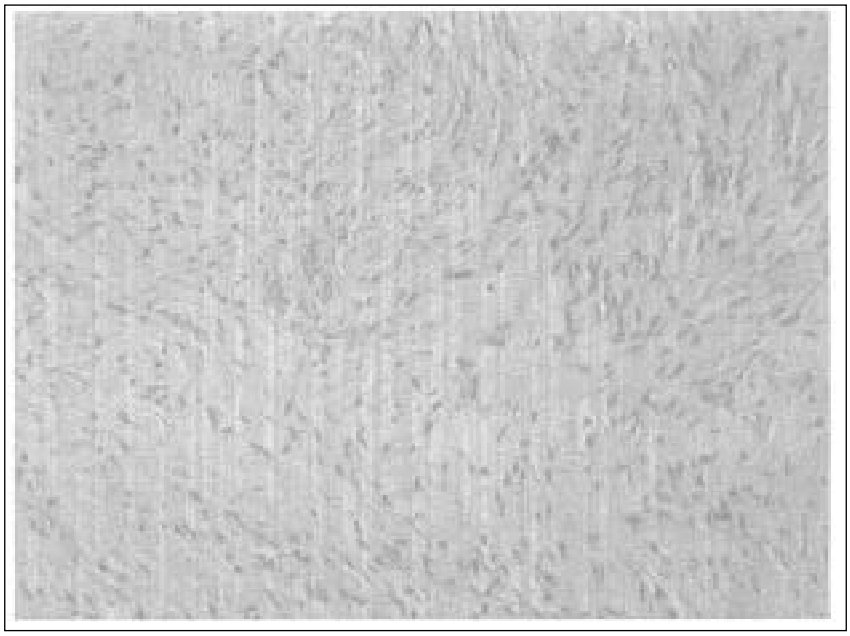
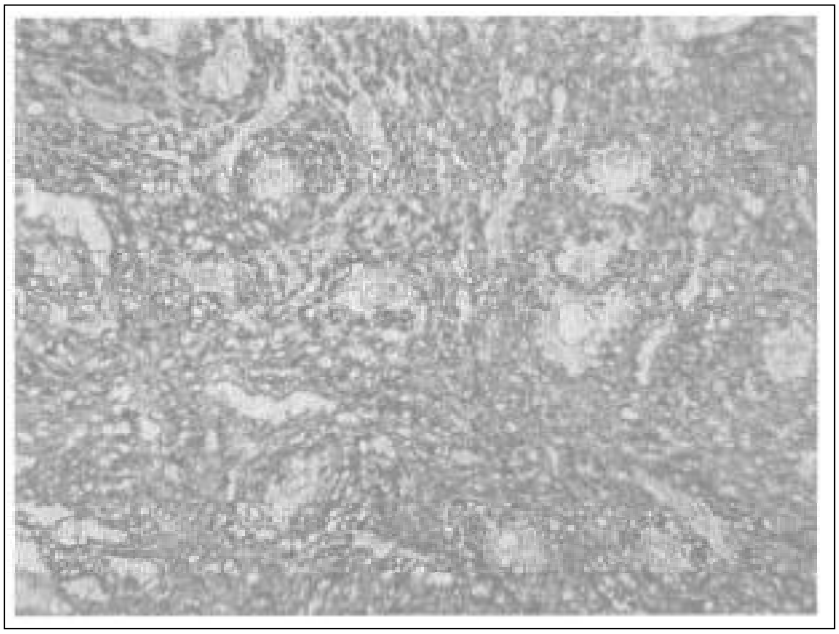


Figure 3 : Schwannoma showing strong and diffuse immunostain for S100 protein.



Diagnostic confirmations can be obtained from genetic studies.

Schwannoma presenting in paediatric age group is rather rare and is especially associated with neurofibromatosis. Symptomatic intraspinal schwannoma is rarely seen in paediatric age group (3). Schwannomas are more commonly found in the intradural, extramedullary space. A study of a large series found that 68.7% of the neurinoma was intradural, 6.1% was extradural, 1.1% intra/ extradural, 5.0% had a dumbbell form, and in 1.1% cases intramedullary, and the remaining cases had neurofibromatosis (4). Schwannoma can present intraosseously and this accounted for less than 0.2% of all primary bone tumors (5). Retroperitoneal localization is rather rare (6).

It is relevant to distinguish between NF1 and NF2 because of their different prognosis. Spinal nerve sheath tumors carry excellent prognosis in NF1 patients and their recurrence rate is very low. On the contrary, symptomatic neurofibromas occurring in NF2 have more severe neurological deficit, poor post-operative recovery and high recurrence rate at 10.7% at 5 years and 28.2% at 10 years respectively (7). This patient recovered well, in keeping with the diagnosis of NF1. All patients with NF1 should receive long-term follow-up for early detection and early intervention if needed in order to prevent irreversible neurological deficits especially when they start exhibiting neurological signs and symptoms. With recent technology, genetic proof is helpful in differentiating between NF1 (chromosome 17q) (8) and NF2 (chromosome 22q) (9) as done in our patient.

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