PACIFIC JOURNAL OF MEDICAL SCIENCES

VOLUME 15, No. 2, JANUARY 2016

PACIFIC JOURNAL OF MEDICAL SCIENCES
{Formerly: Medical Sciences Bulletin}
ISSN: 2072 – 1625

Pac. J. Med. Sci. (PJMS)
www.pacjmedsci.com. Email: pacjmedsci@gmail.com.

ISSN: 2072 – 1625 Volume 15, No. 2, January 2016

A multidisciplinary journal for publication of medical and biomedical research findings on issues pertinent to improving family health and related issues of public health

Pacific Journal of Medical Sciences is officially linked to Asia Pacific Association of Medical Journal Editors (APAME) and listed in the Index Medicus of the Western Pacific Region Index Medicus (WPRIM)

PACIFIC JOURNAL OF MEDICAL SCIENCES

(Formerly Medical Sciences Bulletin)

ISSN: 2072 – 1625 Volume 15, No. 2, January 2016

A multidisciplinary journal for publication of medical and biomedical research findings on issues pertinent to improving family health and related issues of public health

INFORMATION

SUBSCRIPTIONS: Correspondences concerning subscriptions, purchase of single copies and back issues, lost copies and related business should be addressed to the Secretary, Basic Medical Sciences, School of Medicine and Health Sciences, University of Papua New Guinea, P. O. Box 5623 Boroko, N.C.D., PNG. Official website is www.pacjmedsci.com; For Instructions to Authors please visit the official website.

January 2016: ISSN: 2072 – 1625 VOLUME 15, No. 2

TABLE OF CONTENTS Page #

SPECIAL ANNOUNCEMENT

MANILA DECLARATION ON THE AVAILABILITY AND USE OF HEALTH RESEARCH INFORMATION IN AND FOR LOW- AND MIDDLE-INCOME COUNTRIES IN THE ASIA PACIFIC REGION

We, the participants in the Joint Meeting of the Asia Pacific Association of Medical Journal Editors (APAME), the Index Medicus of the South East Asia Region (IMSEAR), and the Western Pacific Region Index Medicus (WPRIM) held in Manila from 24 to 26 August 2015, in conjunction with the COHRED Global Forum on Research and Innovation for Health held in Manila from 24-27 August 2015, drawing on the Pre-Forum Discussions on HIFA from 20 July to 24 August 2015 *"Meeting the information needs of researchers and users of health research in low- and middle-income countries"* available at http://www.hifa2015.org/meeting-the-information-needs-of-researchers-and-users-of-health-research-2/ and the BMJ Blogs 20 July 2015 *"How can we improve the availability and use of health research in developing countries?"* available at http://blogs.bmj.com/bmj/2015/07/20/how-can-we-improve-the-availability-and-use-of-health-research-in-developing-countries/ :

CONSIDERING

That the WHO Constitution "enshrines the highest attainable standard of health as a fundamental right of every human being;" and that "The right to health includes access to timely, acceptable, and affordable healthcare of appropriate quality in tandem with "the underlying determinants of health," including" access to health-related education and information;"

That increasing the availability of quality health research information is fundamental to the successful attainment of global health and progressive realization of the right to health; and that all healthcare stakeholders (individuals, researchers, providers, professionals, leaders and policymakers) need seamless access to peer-reviewed research and information that are relevant to their respective contexts, and presented in a language they can understand;

That despite a growing momentum towards free and open access to research literature, and important initiatives, such as HINARI Access to Research In Health Programme and IRIS Institutional Repository for Information Sharing, that have helped to improve the availability of research in low- and middle-income countries, there continue to be many challenges, limitations and exclusions that prevent health research information from becoming freely and openly available to those who need it;

That the Global Health Library (GHL), Index Medicus of the South East Asia Region (IMSEAR), Western Pacific Region Index Medicus (WPRIM), and Asia Pacific Association of Medical Journal Editors (APAME)are important collaborative initiatives that can promote and uphold the availability and use of health research information especially in and for low- and middle-income countries in the Asia Pacific Region;

CONFIRM

Our commitment to champion and advocate for the increased availability, accessibility and visibility of health research information from and to low- and middle-income developing countries through our Journals, our respective National Associations of Medical Editors, and APAME;

Our commitment to make research information freely and openly available in the right language to producers and users of health research in low- and middle-income countries through IMSEAR, WPRIM, the Asia Pacific Medical Journal Articles Central Archives (APAMED Central) and other platforms;

Our commitment to improve availability, accessibility and interoperability of the different formats of health information suitable to different users in their respective contexts including through both conventional and alternative channels of research dissemination such as new and social media, mobile and disruptive technologies, blogging and microblogging tools and communities, and communities of practice;

CALL ON

Member States of and governments in the South East Asia and Western Pacific Regions, in collaboration with stakeholders from the non-government and private sectors to formulate and implement policies and certification schemes such as the COHRED Fairness Index™ (CFI) that promote free and open availability of health research information for both its producers and users, especially in low- and middle-income countries;

Stakeholders from the public and private sectors, national and international organizations, universities and academic societies, and discussion groups such as Healthcare Information for All (HIFA2015) to support IMSEAR, WPRIM, the GHL, APAMED Central, and develop Integrated Scholarly Information Systems and similar initiatives, in order to ensure the free, open and global accessibility of health research done in the South East Asia and Western Pacific Regions;

The Eastern Mediterranean Association of Medical Editors (EMAME), the Forum for African Medical Editors (FAME), the European Association of Science Editors (EASE), the World Association of Medical Editors (WAME), the International Committee of Medical Journal Editors (ICMJE), the Committee on Publication Ethics (COPE)

and other editors' and publishers' associations to support APAME in implementing various activities, guidelines and practices that would improve the quality, availability and accessibility of scientific writing and publications in the Asia Pacific Region and the world;

Bibliographic, Citation and Full-Text Databases such as PubMed, Global Health Database (CAB Direct), the Directory of Open Access Journals (DOAJ), EMBASE, SciELO Citation Index, Scopus, and the Web of Science to review their policies and processes for indexing Journals from low- and middle-income countries, as well as making health research information freely and openly available to users in these countries who cannot afford to pay for it;

COMMIT

Ourselves and our Journals to publishing innovative and solution-focused research in all healthcare and related fields such as health promotion, public health, medicine, nursing, dentistry, pharmacy, other health professions, health services and health systems, particularly health research applicable to low- and middle-income countries;

Ourselves and our publishers to disseminating scientific, healthcare and medical knowledge fairly and impartially by developing and using Bibliographic Indices, Citation Databases, Full-Text Databases and Open Data Systems including, but not limited to, such Regional Indexes of the Global Health Library as IMSEAR, WPRIM and APAMED Central;

Our organization, APAME, to building collaborative networks, convening meaningful conferences, and organizing participative events to educate and empower editors, peer reviewers, authors, librarians and publishers to achieve real impact, and not just impact factor, as we advance free and open access to health information and publication that improves global health-related quality of life.

26 August 2015, Manila

apame@wpro.who.int

This declaration was launched at the 2015 Convention of the Asia Pacific Association of Medical Journal Editors (APAME) held in Manila from 24 to 26 August 2015. It is concurrently published by Journals linked to APAME and listed in the Index Medicus of the South East Asia Region (IMSEAR) and the Western Pacific Region Index Medicus (WPRIM).It is co-published with special permission in the Pacific Journal of Medical Sciences that was represented in the APAME2015 Convention and Joint Meeting with the Western Pacific Region Index Medicus Regional Journal Selection Committee Meeting.

Additional Information:

Indexing of the Pacific Journal of Medical Sciences:

The Pacific Journal of Medical Sciences (Pac. J. Med. Sci.) participated in the 2015 Convention of the Asia Pacific Association of Medical Journal Editors (APAME) held in Manila Philippines from 24 to 26 August 2015. The Managing Editor of the Pacific Journal of Medical Sciences was enrolled as a member of the APAME during the APAME 2015 Convention and Joint Meeting with the Western Pacific Region Index Medicus Regional Journal Selection Committee Meeting. **The Pacific Journal of Medical Sciences is now officially recognised as one of the several Journals linked to APAME and listed in the Index Medicus of the Western Pacific Region Index Medicus** (**WPRIM**).

Journals indexed on WPRIM and IMSEAR may co-publish the Manila declaration as a **Special Announcement**, with the statement: "This declaration was launched at the 2015 Convention of the Asia Pacific Association of Medical Journal Editors (APAME 2015) held in Manila from 24 to 26 August 2015 in conjunction with the COHRED Global Forum on Research and Innovation for Health (FORUM 2015). It is concurrently published by Journals linked to APAME and listed in the Index Medicus of the South East Asia Region (IMSEAR) and the Western Pacific Region Index Medicus (WPRIM). It is co-published with special permission in the **Pacific Journal of Medical Sciences** that was represented in the APAME2015 Convention and Joint Meeting with the Western Pacific Region Index Medicus Regional Journal Selection Committee Meeting."

SEASONAL VARIATION IN SECONDARY SEX RATIO IN EDO STATE, NIGERIA: A RETROSPECTIVE STUDY

Alphonsus N. **Onyiriuka** and Eugene M. **Ikeanyi**

*^Endocrine and Metabolic Unit, Department of Child Health, University of Benin Teaching Hospital, Benin City, Nigeria; **Department of Obstetrics and Gynaecology, College of Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

^Corresponding author: alpndiony@yahoo.com

ABSTRACT:
Season is known to influence human secondary sex ratio but information on this subject is lacking in Nigeria. The objective of this study was to determine the secondary sex ratio (SSR) during the wet and dry seasons in Nigeria. In this retrospective cohort study, the records of all deliveries at St Philomena Catholic Hospital (SPCH), Benin City, Edo State, Nigeria between 1st January, 2005 and 31st December, 2014 (10 years) were retrieved and analyzed. The births were recorded according to the year and month of delivery. Stillbirths and infants with ambiguous genitalia were excluded from the analyses. The total number of live-births during the 10-year period under review was 13,702 and this consisted of 7,007 males and 6,695 females, resulting in a secondary sex ratio of 104.6:100 (1.05:1). In general, the monthly distribution of births was bimodal with a greater peak in May and a lesser peak in October with the highest and lowest SSRs in the months of June and March, respectively. In the dry season, the proportion of male births was higher than the proportion of female births; $p > 0.05$. In contrast, in the wet season the proportion of male births was lower than the proportion of female births; $p > 0.05$. In addition, the SSR was higher in the dry season (1.04) compared with the wet season (0.99). In Edo State, Nigeria, the proportion of male births tended to be higher than the proportion of female births during the dry seasons.

Key words: Dry season, seasonality of births, secondary sex ratio, wet season, Nigeria.
Submitted: October 2015, Accepted: December 2015

INTRODUCTION:

The phrase “secondary sex ratio” is defined as the number of male live-births for every 100 female births [1]. The close proximity of Nigeria to the equatorial belt and the Atlantic Ocean confers on her a humid tropical climate with two main seasons. The seasons are the wet (rainy) and the dry seasons [2]. In Nigeria, the average annual rainfall varies from 1,770mm (70 inches) in the west to 4,310mm (170 inches) along the east coast and to 470mm (50 inches) in the central areas. The average temperature range all year round is 23 to 32 degrees Celsius (73 to 90 degrees Fahrenheit) [2]. In almost all human populations, the number of births varies by season of the year but this variation is not identical in all populations [3]. It has been documented that the most important environmental factors that influence the monthly distribution of births are seasonal variation in temperature [4] and rainfall [5]. The mechanisms by which any variable that brings about seasonal

changes in birth pattern operates include sperm quality, sexual activity, seasonal pregnancy loss, seasonal availability of food and cultural factors [3,5,6]. Observations in Hong Kong, Bangladesh, Uganda and Singapore indicate that a negative correlation exist between environmental temperature and the number of monthly conceptions and this has been attributed to hormonal changes [6]. Intra-population variation in sex ratio has been linked to season of conception and by extension, the season of birth [7-10]. In the study by Helle et al [8], fluctuations in rainfall and temperature influenced human secondary sex ratio (SSR). It has been proposed that climate may cause a physiological stress and thus, play a role in sex allocation in humans. In this regard, it may affect the SSR if the mothers are exposed to shifts in temperature during gestation [11]. Evolutionary theory predicts that fewer males should be born during stressful periods, as a weaker male will not survive to reproduce where a female might [12]. The report by McLchlan and Storey suggested that more males would be born during the warmer periods [9]. Studies in Finland and other Scandinavian countries found that more males are born in warmer years [11,13]. In addition, increases in temperature increase the likelihood of a male fetus surviving [11]. In contrast, a study in New Zealand on the effects of fluctuations in mean annual temperature found that it was unrelated to rates of male births [14].

SSR differs from one population to the other. This variation has been attributed to various factors including season. In this regard, it might be useful to examine the influence of season (a natural factor) on the likelihood of the birth of a male child. The knowledge gained might find an application in the reproductive health of families, allowing for some degree of parental choice of sex of offspring. To the best of our knowledge, there are no previous published studies that have assessed the relationship between season and SSR in Nigeria. In most cultures in Nigeria, parents depend on their sons for immediate status and future security [15,16]. The resultant effect is that the birth of a male child is more celebrated than that of a female child [15]. These considerations prompted the present study. The purpose of the study is to determine the secondary sex ratio during the wet and dry seasons in Edo State, Nigeria.

SUBJECTS AND METHODS:

A retrospective audit of the birth records of all deliveries at St Philomena Catholic Hospital (SPCH) between 1st January, 2005 and 31st December, 2014 (10 years) was carried out. The hospital has a large maternity unit (ranks second in Edo State) located at the centre of Benin City, Edo State, Nigeria and it is easily accessible. Stillbirths were excluded in the analysis in conformity with the definition of secondary sex ratio. Infants with ambiguous genitalia were also excluded from the analysis. The deliveries were recorded according to the month and year of delivery. The study design was approved by the hospital authority. The secondary sex ratio was

computed, using the formula B/G (where 'B' is number of male births and 'G' is number of female births). In this study, the seasons were defined as wet (rainy) season May to October and dry season November to April.

Statistical analysis was performed using the statistical package for social sciences (SPSS, version 16.0). Descriptive statistics such as frequencies, ratios, and percentages were used to describe all the variables. The Z-test was used in ascertaining the significance of differences between two proportions with $p < 0.05$.

RESULTS:

During the 10 years covered in this study, a total of 13,702 live-births were recorded at SPCH and this consisted of 7,007 males and 6,695 females. Males accounted for 51.1% of the total live-births. A quarter (24.9%) of the total births was recorded in the years 2013 and 2014. A total of 6,703 (48.9%) and 6,999 (51.1%) babies were born during the dry and wet seasons, respectively. The overall secondary sex ratio (SSR) for the 10 years pooled together was 1.05:1.0. Table 1 shows the monthly distribution of births and SSRs for the 10 years period. The cumulative monthly distribution of births was bimodal with a greater peak in May and a lesser peak in October as can be seen in Figure 1. The highest and lowest SSRs were in the months of June and March, respectively. As depicted in Table 2, the proportion of male births was higher than the proportion of female births during the dry season ($p > 0.05$). In the wet season, the proportion of male births was lower than female births ($p > 0.05$). In addition, the SSR was higher in the dry season (1.04) compared with the wet season (0.99).

Table 1: Monthly cumulative number of live births, gender distribution and secondary sex ratios from 2005 to 2014

Months of study (2005-2014)	Total by month of study (2005-2014)	Number of males	Number of females	Secondary sex ratio
January	1124	571	553	1.03
February	967	498	469	1.06
March	1199	595	604	0.98
April	1151	599	552	1.09
May	1371	712	659	1.08
June	1139	599	540	1.11
July	1199	598	601	0.99
August	1106	569	537	1.06
September	1156	594	562	1.06
October	1262	643	619	1.04
November	960	499	461	1.08
December	1068	530	538	0.99
Jan-Dec	13702	7007	6695	1.05

Figure 1: Monthly cumulative number of live births and gender distribution of births from 2005 to 2014

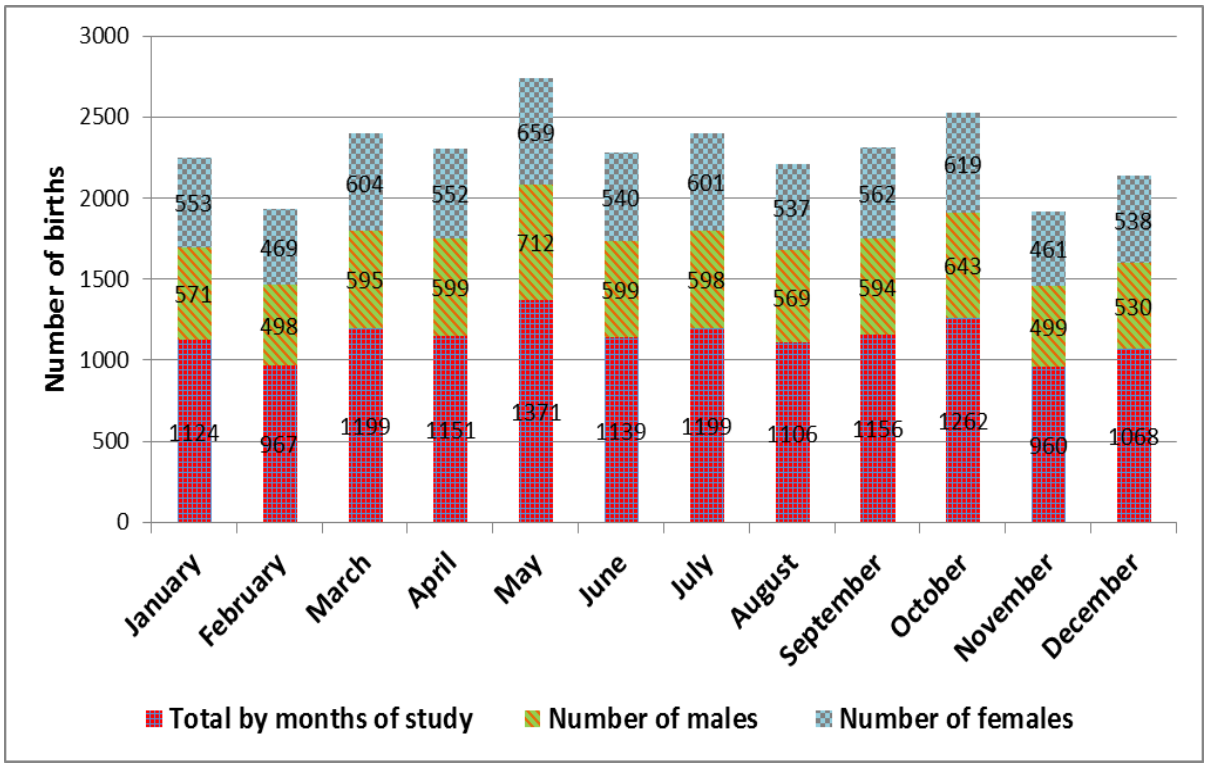


Table 2: Yearly distribution of secondary sex ratio (SSR) of live births in the period 2005 to 2014

Year of study	Yearly total	Season of the year					
		Dry season (n=6703)			Wet season (n = 6999)		
		Males	Females	SSR	Males	Females	SSR
2005	1128	312	310	1.01	252	254	0.99
2006	1487	363	350	1.04	386	388	0.99
2007	1304	277	274	1.01	375	378	0.99
2008	1230	316	311	1.02	303	300	1.01
2009	1304	295	284	1.04	357	368	0.97
2010	1332	285	286	1.00	379	382	0.99
2011	1105	275	244	1.13	294	292	1.01
2012	1398	379	366	1.04	325	328	0.99
2013	1653	436	415	1.05	399	403	0.99
2014	1761	472	453	1.04	420	416	1.01
2005-2014	13,702	3410	3293	1.04	3490	3509	0.99
SSR				1.04			0.99
Percentage		50.9%	49.1%		49.9%	50.1%	
Z-statistic		0.98; p > 0.05			1.00; p > 0.05		

DISCUSSION:

Although the data from our study showed that a higher proportion of male births compared with female births occurred in the dry season, the difference was not statistically significant. In contrast, during the wet season, the proportion of male births was lower than the proportion of female births. Again, this difference was not statistically significant. In general, these findings suggest that there was a greater tendency to deliver male babies during the dry season compared with during the wet season. No published similar study from Nigeria for comparison. However, the results of the studies by several authors indirectly supported our finding [9,11,13]. In contrast, our results contradict the findings in New Zealand [14]. Maconochie and Roman did not find any evidence that season influenced secondary sex ratio [17]. In that study, they concluded that gender determination is purely a chance process. The reason for the different finding is not clear. However, it suggests that other factors, apart from season, influence SSR. Various mechanisms have been postulated to explain the influence of season on SSR. These include the influence of temperature on coital frequency and hormonal changes. The coital frequency is known to vary with season with a lower rate during the hot weather [18]. Infants conceived during the dry season when coital frequency is relatively lower are usually delivered during the wet season. Similarly, infants conceived during the wet season when there is higher coital frequency are usually delivered in the dry season. A lower coital rate elongates the average interval between ovulation and fertilization (the German hypothesis), causing overripeness ovopathy [19]. The resultant effect may be an increased likelihood of chromosomal anomalies and spontaneous abortions [19,20]. Male fetuses are known to be more vulnerable, leading to a greater loss and subsequently, a lower proportion of male births [19]. Similarly, a lower coital frequency causes ageing of the spermatocytes, resulting in spermatopathy [20,21]. Ageing Y-bearing spermatocyte may move more sluggishly, reducing the chances of fertilizing an ovum [21]. The resultant effect of the above considerations is a relatively higher male birth rate during the dry season compared with during the wet season. In addition, during the wet seasons of the year, spontaneous abortions induced by infections (e.g., malaria) are more likely and these miscarriages are often unknown to women and so, are unreported to interviewers [22]. Male fetuses are more vulnerable to such abortions.

Our data indicate that the cumulative monthly distribution of births is bimodal with the greater peak in May and the lesser peak in October with the troughs in the months of February and November. In a multicentre (Obafemi Awolowo University Teaching Hospital, Ile Ife, Wesley

Guild Hospital, Ilesa, General Hospital, Ogbomosho and Ekiti State Specialist Hospital, Ado-Ekiti) study, a similar cumulative monthly distribution of births from 1995 to 2004 was found on analysis of the data involving 14,579 live births subjects derived from Obafemi Awolowo University Teaching Hospital, Ile Ife, Nigeria [23]. However, in the same study, data from the remaining three other hospitals with lower delivery rates, the cumulative monthly distribution of births were different, suggesting that the monthly delivery rate might influence the monthly SSRs. The reason for this disparity is not clear. In our study, we found that the highest SSR was in the month of June. In consonance with our finding, Azeez et al [23], also reported that the highest SSR was in the month of June. On the other hand, we found that the lowest SSR was in the month of March whereas Azeez et al [23] found that the lowest SSR was in the month of October. Overall, the monthly SSR during the period covered by our study did not reveal a regular pattern of variation. This observation is similar to a previous study in Nigerian which reported a similar finding [23]. In that study involving four different hospitals in southwest Nigeria, the highest and lowest SSRs according to the month of birth were different for each of the four hospitals, illustrating the wide variability in monthly distribution of live births.

ACKNOWLEDGEMENTS:

We are grateful to the Chief Medical Officer of the hospital for granting us access to the hospital's delivery records. We thank all members of staff in the Records section for making the delivery registers used in this study available.

Duality of interest: No conflict of interest with regard to this study.

REFERENCES:

1. Hesketh T, Xing WZ. Abnormal sex ratio in human populations: causes and consequences. Proc Natl Acad Sci, USA 2006;103(36):13271-13275.
2. Anonymous. Nigeria-Atlapedia Online. Available www.atlapedia.com/online/countries/nigeria.htm. Accessed on May 20, 2015.
3. Bobak M, Gjonca A. The seasonality of live birth is strongly influenced by sociodemographic factors. Hum Reprod 2001;16(7):1512-1517.
4. Lam DA, Miron JA. Temperature and the seasonality of births. Adv Exp Med Biol 1991;286:73-88.
5. Bronson FH. Seasonal variation in human reproduction: environmental factors. Q Rev Biol 1995;70(2):141-164.
6. Dyson T, Crook N. Causes of seasonal fluctuation in vital events. Available at: www.popline.org/node/390563. Accessed on June 1, 2015.
7. Lerchl A. Seasonality of sex ratio in Germany. Hum Reprod 1998b; 13:1401-1402.
8. Helle S, Helama S, Lertola K. Evolutionary ecology of human birth sex ratio under the compound influence of climate change, famine, economic crises and wars. J Animal Ecology 2009;78:1226-1233.
9. McLachlan TC, Storey H. Hot male: can sex in humans be modified by temperature? J Theor Biol 2003;222:71-72.
10. Kumari JR, Rao TV. Seasonal variation of human sex ratio at birth and the modifying factors. Indian J Pediatr 1982;49(4):541-546.

11. Catalano R, Brucker T, Smith KR. Ambient temperature predicts sex ratio and male longevity. Proc Natl Acad Sci USA 2008;105:2244-2247.
12. Trivers RL, Willard DE. Natural selection of parental ability to vary the sex ratio of offspring. Science 1973;179:90-92.
13. Helle S, Helama S, Jokela J. Temperature-related birth sex ratio bias in historical Sami: Warm years being more sons. Biol Letters 2008;4:60-62.
14. Dixson BJ, Haywood J, Lester PJ, Ormsby DK. Whatever the weather: Ambient temperature does not influence the proportion of males born in New Zealand. PLosONE 6(9):e25064.
15. Nnadi I. Son preference - A violation of women's human rights: A case study of Igbo custom in Nigeria. J Politics Law 2013;6(1):134-141.
16. Milazzo A. Son preference, fertility and family structure: evidence from reproductive behaviour among Nigerian women. Policy Research Working Paper; no. WPS 6869. Washington, DC: World Bank Group, 2014.
17. Maconochie N, Roman E. Sex ratio: are there natural variations within the human population? Br J Obstet Gynaecol 1997;104:1050-1053.
18. Athanassena GA. Seasonal variation of births in Greece: Temporal changes and regional differences over a 24-year period. Chronobiologia 1985;12:351.
19. German J. Mongolism, delayed fertilization and human sexual behavior. Nature 1968;217:516.
20. Guerrero V, Rojas OI. Spontaneous abortion and aging of human ova and spermatozoa. New Engl J Med 1987;293:573.
21. Reijneveld SA, De Bock GH. Seasonal preovulatory overripeness ovopathy: A reappraisal of the concept. Med Hypothesis 1992;37:151-157.
22. Pitt MM, Sigle W. Seasonality, weather shocks and timing of births and child mortality in Senegal. A paper presented at the 10th anniversary conference of the Centre for Study of African Economies, Oxford University, Oxford, England, April 17-18, 1997.
23. Azeez MA, Akinboro A, Bakare AA. Human sex ratio at birth in southwest Nigeria. Indian J Hum Genet 2007;13(2):59-64.

EFFECT OF SMOKING ON GINGIVAL KERATINOCYTE PROLIFERATION IN SMOKERS AND NON-SMOKERS USING IMMUNOHISTOCHEMISTRY (PCNA)

***Swati Penumarthy, **Ravikanth Manyam, ^Sesha Reddy Manchala, *Narendra Babu Mandalapu, *CD Dwrakanath and *Gauthami S Penumatsa**

***Department of Periodontics and **Department of Oral and Maxillofacial Pathology, Vishnu Dental College, Vishnupur, Bhimavaram, WG Dt, Andhra Pradesh.**
^College of Dentistry, Gulf Medical University, UAE

^Corresponding Author: seshaperio5@gmail.com

Running Title: *Expression of PCNA in Oral Gingival Epithelium of smokers and nonsmokers gingiva*

ABSTRACT:
Tobacco smoking is one of the major risk factors for periodontal diseases. Prolonged and heavy smoking can reduce gingival bleeding and therefore mask the clinical marker of bleeding on probing. Reduced gingival bleeding occurs in smokers than nonsmokers due to vasoconstriction of gingival vessels; it may also be attributed to the heavier keratinization of the gingiva in smokers. Although it is well established that smoking increases the proliferation of cells in healthy and dysplastic oral mucosa, the effect of smoking on the proliferative activity of gingival keratinocytes has not been extensively evaluated in periodontally disease patients. Therefore, the aim of our study was to determine the effects of smoking on the proliferation of gingival epithelium in subjects with gingivitis and those with periodontitis immunohistochemically using monoclonal antibodies to proliferating cell nuclear antigen (PCNA).

Key Words: Immunohistochemistry, Mitotic Index, Proliferating Cell Nuclear Antigen, Smokers gingiva, Non-smokers gingiva.

Submitted: October 2015, Accepted: December 2015

INTRODUCTION:

Periodontal disease is an infectious disease resulting in inflammation within the supporting tissues of the teeth with progressive attachment loss and bone loss [1]. A clear association has been demonstrated between smoking and periodontal disease [2,3]. However the clinical signs of inflammation such as bleeding on probing and gingival inflammation are masked in smokers than non-smokers [4]. This could be due to nicotine and cotinine which are the components of cigarette. Nicotine stimulates the

sympathetic ganglia to produce neurotransmitters including Catecholamines affecting the α-receptors on blood vessels which in turn cause vasoconstriction whereas cotinine a nicotinic metabolic byproduct has a peripheral constrictive action on gingival vessels. The other possible reason could be due to the heavy keratinization of the gingiva in smokers [4,5].

The prevalence, severity of periodontal destruction and tooth loss is increased in smokers compare to non-smokers, which is due to altered neutrophil chemotaxis, phagocytosis and oxidative burst[6-9] There is also an increase in inflammatory components TNF-α, PGE2, collagenase and elastase in Gingival crevicular fluid (GCF) of smokers when compared to non-smokers. Nicotine stimulates IL-1α synthesis by human gingival keratinocytes because of which there is more periodontal destruction. Smokers have more pathogenic microflora but smoking has no effect on plaque accumulation [10].

Outer epithelial surface of gingiva is inflexible, tough and resistant to abrasion resulting from the formation of surface layer of keratin. This keratinization helps in forming an impermeable barrier thus limiting the penetration of toxins and antigens produced by microorganisms present in oral cavity. The permeability barrier is believed to consist of lipids derived from the membrane coating granules and the tight junctions formed by the intimate association of keratohyalin granules with tonofibrils [11]. Nicotine was shown to induce the proliferation of oral keratinocytes because it displaces the local cyto-transmitter acetylcholine from the nicotinic receptor (nAchRs) expressed by oral keratinocytes [12]. Van Oijen et al speculated that the increased proliferation index in smokers was due to the regenerative effect that was explained by the local regenerative response to compensate for increased cell loss or damage by tobacco as well as the other protracted effects of smoking [13]. Thickening of the gingival epithelium and rete proliferation could be due to an imbalance in homeostasis between cell division and cell loss [14,15].

The proliferative activity of the oral mucosal and gingival epithelium has been analyzed for many years by monoclonal antibodies to agents as proliferating cell nuclear antigen (PCNA). It is one of the nuclear antigens; it is a 36 KDa auxiliary protein to DNA polymerase delta. It is expressed in the nuclei of cells during the DNA synthesis phase of cell cycle. PCNA is clamped to DNA through the action of replication factor c (RFC) which is a hetero-pentameric member of the AAA+ class of ATPases. Expression of PCNA is elevated in the nucleus during the late G1 phase, immediately before the onset of DNA synthesis; expression becomes maximal during S phase before declining during the G2 and M phases. The level of PCNA correlates directly with rates of cellular proliferation and DNA synthesis [16].

Prolonged heavy smoking can reduce gingival bleeding and therefore mask the clinical marker of bleeding on probing often used by dentists to

monitor periodontal health. Reduced gingival bleeding in smokers than non-smokers due to vasoconstriction of gingival vessels may also be attributed to the heavier keratinization of the gingiva in smokers [17]. Therefore, the aim of our study was to determine the effects of smoking on the proliferation of gingival keratinocytes in subjects with gingivitis and those with periodontitis immunohistochemically using monoclonal antibodies to proliferating cell nuclear antigen (PCNA).

SUBJECTS AND METHODS:

A total of 60 subjects from the patient pool attending the outpatient clinic, Department of Periodontics, Vishnu Dental College and Hospital were enrolled in the study. The institutional ethical committee and review board approved the study protocol. Informed consent was obtained from each patient. Subjects exhibiting good general health with history of smoking with no history of periodontal treatment or antimicrobial therapy for the past 6 months were selected.

Subjects were placed into 6 groups of 10 subjects each. Non-smokers with clinically healthy gingiva (NSH; n=10), non-smokers with gingivitis (NSG; n=10), non-smokers with periodontitis (NSP; n=10), smokers with clinically healthy gingiva (SH; n=10), smokers with gingivitis (SG; n=10) and smokers with periodontitis (SP; n=10). The groups NSH and SH were recruited as the control with a gingival index score of 0 for the non-smoker and smoker groups respectively. The criteria for gingivitis were no attachment loss and a gingival index of >1. Periodontitis tissues were collected during modified Widman flap technique by giving internal bevel incision[18] The criteria for selecting smokers was individuals who had smoked 100 or more cigarettes over their life time and smoked at the time of interview (current smokers – Centers for disease control and prevention CDC).

Tissue collection:

All gingival biopsies (one per person) were collected. Biopsy samples were taken from the marginal gingiva in healthy and gingivitis groups during crown lengthening procedures and from periodontitis group during periodontal surgery and were fixed in 10% neutral buffer formalin and paraffin blocks were prepared.

Immunohistochemistry:

The tissues were deparaffinized by two changes in xylene (10 minutes) each, put in descending grades of alcohol and then rehydrated with water. They were then transferred to citrate buffer and antigen retrieval was done using microwave method 3 cycles 5 min each at power 450W. Then the slides were dipped in two changes of phosphate buffered saline (PBS) for 5 minutes and then wiped carefully with gauge to remove excess PBS. The slides were treated with 3% hydrogen peroxide for 10 minutes, and put in two

changes of PBS and then treated with power block for 20 minutes [19].

The primary antibody PC10 (Bio-Genex) were added to tissue samples and was incubated at room temperature for 1 hour. After one hour, the sections were taken out and washed in two changes of cold PBS (10minutes each), a drop of super enhancer was added and the slides were incubated for 30 minutes. The slides were then washed in two changes of PBS 10 minutes each. Then the secondary antibody was added and incubated for 30minutes. Then the sections were washed and carefully wiped to remove excess PBS and a drop of freshly prepared Diaminobenzidine (DAB) as added onto sections. Slide were then washed in running distilled water to remove excess DAB and counter stained with Harri's hematoxylin. They were washed with acid alcohol and xylene. The tissue sections were mounted with disterene dibutylphthalate (DPX) for microscopic examination at magnification 40x [19].

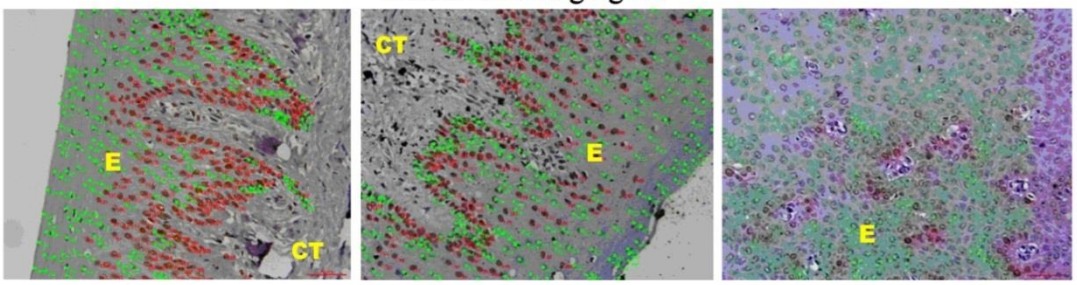
Evaluation:

The images were captured using BX 51 microscope with 0.5 adapter, 40X lens and DP 71 camera. 100 cells from the basal and parabasal layer were counted. The slide was divided into 5 grids and PC 10 expression was evaluated by counting the number of cells that were stained in brown color. The proliferation index was calculated by dividing the number of positively stained cells with the total number of cells in the magnified section. Data entry and descriptive analysis was performed using the SPSS statistical software program. Statistical analysis using paired T-Test was done.

RESULTS:

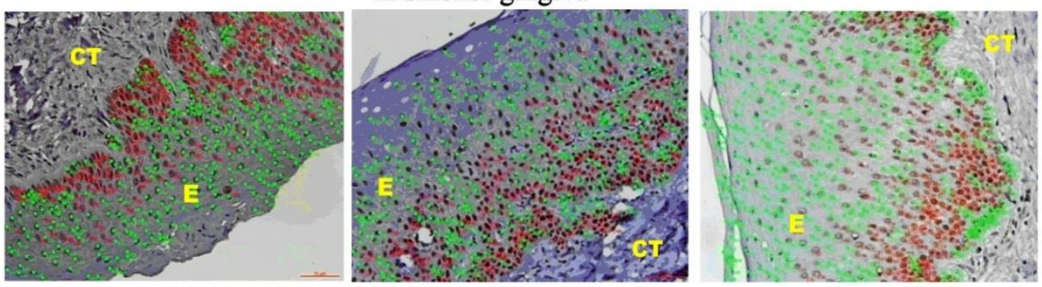
The proliferation index in non-smokers and smokers are shown in Figures 1. and 2. Cells with red nuclei were considered positively stained for PCNA. PCNA positive cells showed a uniformly dense red staining of nuclei, observed in basal and parabasal layers of the gingival epithelium. Highest numbers of PCNA positive cells are seen in smokers with healthy gingiva, least in non-smokers with periodontitis. Table1 shows comparison of proliferation index values within the non-smoker groups. Proliferation index values were statistically significant in NSH and NSG group compared to NSP group (P value < 0.05). Statistically significant difference was not observed between NSH and NSG groups. Table 2 shows comparison of proliferation index values within the smoker groups. Significant difference in the proliferation index values were seen in SH and SG group when compared to SP group (P value less than 0.05). However, no statistically significant difference was observed between SH and SG groups. Table-3 and figure-3 show highest proliferation index value in smokers gingiva compared to non-smokers gingiva (P value < 0.05)

Fig - 1 : PCNA staining in epithelium and connective tissue after cell counting in non-smoker gingiva



a - Normal gingiva b - Gingivitis c - Periodontis

Fig - 2 : PCNA staining in epithelium and connective tissue after cell counting in smoker gingiva



a - Normal gingiva b - Gingivitis c - Periodontis

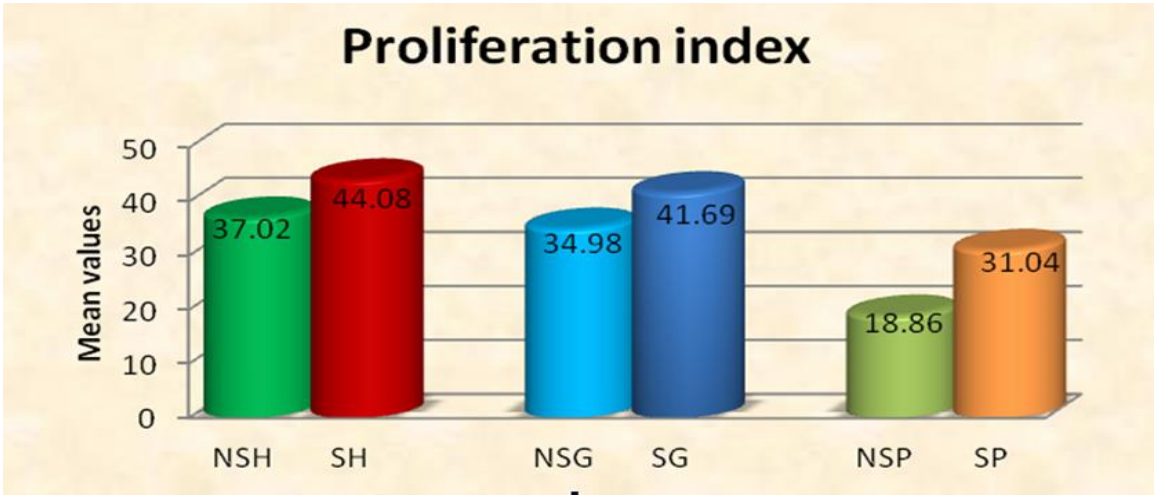


Figure 3: Proliferation index in smokers and nonsmokers gingiva
NSH: Non-Smokers with clinically Healthy gingiva; NSG: Non-Smokers with Gingivitis
NSP: Non-Smokers with Periodontitis; SH: Smokers with clinically Healthy gingiva
SG: Smokers with Gingivitis; SP: Smokers with Periodontitis

Table-1: PCNA INDEX Comparison within smoker and non-smoker groups

	N	Mean ± S.D	P VALUE
NSH	10	37.02 ± 5.44	0.044 Significant
SH	10	44.08 ± 5.99	
NSG	10	34.98 ± 4.79	0.007 Significant
SG	10	41.69 ± 7.42	
NSP	10	18.86 ± 10.07	0.019 Significant
SP	10	31.04 ± 8.65	

Statistically significant, if $p<0.05$

Table-2: Comparison between SH&SG, SH&SP and SG&SP.

	N	Mean ± S.D	P VALUE
SH	10	44.08 ± 5.99	0.417 Not Significant
SG	10	41.69 ± 7.42	
SH	10	44.08 ± 5.99	0.001 Significant
SP	10	31.04 ± 8.65	
SG	10	41.69 ± 7.42	0.012 Significant
SP	10	31.04 ± 8.65	

Statistically significant, if $p<0.05$

Table-3: PCNA index Comparison within non-smoker groups.

	N	Mean ± S.D	P VALUE
NSH	10	37.02 ± 5.44	0.331 Not Significant
NSG	10	34.98 ± 4.79	
NSH	10	37.02 ± 5.44	0.003 Significant
NSP	10	18.86 ± 10.07	
NSG	10	34.98 ± 4.79	0.002 Significant
NSP	10	18.86 ± 10.07	

Statistically significant, if $p<0.05$

DISCUSSION:

The present study revealed that proliferation index is higher in healthy group compared to gingivitis and periodontitis group. Table1 shows a significant difference in the proliferation index values between group NSH & NSP and NSG & NSP with a P value of <0.05 (figure3), which is mainly due to the inhibitory effect of infection on the proliferation of keratinocytes. Jarnbring et al [20] demonstrated fewer PC10 positive keratinocytes in the periodontitis group. This is due to the cell composition of the inflammatory infiltrate. T-cell and B-cell infiltrates could have both a negative effect on proliferation and a stimulating effect on apoptosis. T-cell products interferon-γ (IF-γ) and transforming growth factor-β (TGF-β) are both inhibitory for keratinocyte proliferation. Cytokines such as interleukin-4 (IL-4) and TGF-β have been shown to stimulate cell expression of P53 and promote apoptosis in the cell [20]

The proliferation index is highest for smokers with healthy gingiva (44.08 ± 5.99) table 2. The mean values were more in smoker group compared to non-smokers. Smoking alone had an effect on the proliferation of cells in the oral gingival epithelium regardless of the periodontal status of the subjects. Nicotine was shown to induce the proliferation of oral keratinocytes figure 4. Arredendo J et al [12] demonstrated that chronic stimulation of oral keratinocytes by nicotine alters the genetically determined program of the cell differentiation dependent expression of nAchRs subunits. Exposure of keratinocytes to nicotine also altered the mRNA and protein levels of cell cycle and cell differentiation marker PCNA. Therefore the downstream signaling from nAchRs expressed in the oral mucosa proceed via a pathway that up regulates the expression at transcriptional and translational levels of cell cycle progression regulators [12]. The findings of the present study are in accord with the study carried out by da Costa Filho et al [21] who detected an increase in the PCNA index in the gingival epithelium of smokers compared to non-smokers.

Analysis of the available data suggests that smokers may have a more pathogenic microflora. In that Bacteroides forsythus harbors subgingivally more in smokers than in non-smokers [22]. There was a tendency for Porphyromonas gingivalis, Prevotella intermedia, Eikenella corrodens, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans counts to be higher in smokers than non-smokers [23]. Plasma responses of smokers following lipopolysaccharide stimulation differed from those of non-smokers, in that smokers had significantly more TNF-α [24], IL-6, α2-macroglobulin, IL-1, IL-8 and monocyte chemotactic protein-1 levels [2]. This suggests that smokers have more severe disease compared to non-smokers. In this respect presence of high cell turnover in the keratinocytes, which are the major cell type of the

gingival epithelium is important for establishing and maintaining host-microorganism equilibrium. This may be the fact for higher mitotic index values in smokers compared to non-smokers in the present study.

Table 3 shows the mean values of proliferation index in non-smokers and smokers. The mean values were high in healthy group (NSH=37.02 ± 5.44, SH=44.08 ± 5.99) compared to gingivitis group (NSG= 34.98 ± 4.79, SG=41.69 ± 7.42) and periodontitis group (NSP=18.86 ± 10.07, SP=31.04 ± 8.65) figure 5. The results of the present study are supported by Gultekin SE et al [25] who demonstrated highest proliferation index for smokers with healthy gingiva and least for non-smokers with periodontits.

CONCLUSION:

Smoking induced the proliferation of gingival keratinocytes, regardless of the periodontal status of the subjects, because a significant increase in proliferation index was found in smokers compared to non-smokers. This heavy keratinization of gingiva in smokers can mask clinical signs of inflammation and reduce gingival bleeding which is an indicator of active tissue destruction. Hence clinical attachment level and probing pocket depth are reliable clinical findings for assessment of periodontal destruction in smokers.

REFERENCES:

1. Offenbacher S. Periodontal diseases: Pathogenesis. Ann Periodontol 1996; 1:821-878.
2. Kinane DF, Chestnutt IG. Smoking and periodontal disease. Crit Rev Oral Biol Med 2000; 11:356-365.
3. Bergstrom J, Preber H. Tobacco use as a risk factor. J Periodontol 1994; 65:545-550.
4. Dietrich T, Bernomoulin JP, Glynn RJ. The effect of cigarette smoking on gingival bleeding. J Periodontol 2004; 75:16-22.
5. Pejcic A, Obradovic R, Kesic L, Kojovic D. Smoking and periodontal disease a review. Medicine and biology 2007; 14:53-59.
6. Haber J, Waltles J, Crowley M, Mandell R, Joshipura K, Kent RL. Evidence for cigarette smoking as a major risk factor for periodontitis. J Periodontol 1993; 64:16-23.
7. Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R et al. Assessment of Risk for Periodontal Disease. II. Risk Indicators for Alveolar Bone Loss. J Periodontol 1995; 66:23-29.
8. Bergstrom J, Eliasson S, Dack J. A 10-year prospective study of tobacco smoking and periodontal health. J Periodontol 2000; 71:1338-1347.
9. Calsina G, Ramon JM, Echeverria JJ. Effect of smoking on periodontal tissues. J Clin Periodontol 2002; 29:771-776.
10. Johnson GK, Organ CC. Prostaglandin E2 and interleukin-1 concentration in nicotine exposed oral keratinocyte cultures. J Periodont Res 1997; 32:447-454.
11. Schroeder HE, Listagarten M, The gingival tissues: the architecture of periodontal protection. Periodontology 2000, 1997, 13:91-120.
12. Arrendondo J, Chernyavsky AI, Marubio LM, Beaudet AL, Jolkovsky DL, Pinkerton KE et al. Receptor mediated tobacco toxicity. Regulation of gene expression through alpha 3 beta 2 nicotinic receptor in oral epithelial cells. Am J Pathol 2005; 166:597-613.
13. Van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. Oral Oncol 1998; 34:297-303.
14. Villar CC, de Lima AF. Smoking influences on the thickness of marginal gingival

epithelium. Presqui Odontol Bras 2003; 17:41-45.
15. Bajagic V, Pejcic A, Zivkovic V, Petrovic A. Histochemical study of gingival epithelium in smokers and non-smokers. Acta Fac Med Naiss 2006; 23:151-154.
16. Kelman Z. PCNA: structure, function and interactions. Oncogene 1997; 14: 629-640.
17. Scott DA, Singer DL. Suppression of overt gingival inflammation in tobacco smokers-clinical and mechanistic considerations. Int J Dent Hygiene 2004; 2:104-110.
18. Ramfsford SP, Nissle RR, The modified widman flap.J Periodontol 1974 Aug;45(8):601-7.
19. Jyostna P, Reddy MS, Ravjkanth M, Narendra M, Dwarakanth CD. Expression of PCNA in oral gingival epithelium of aggressive and, chronic periodontitis—A pilot study.OJST2013.Vol. 3,No1,112 – 117.
20. Jarnbring F, Somogyi E, Dalton J, Gustafsson A, Klinge B. Quantitative assessment of apoptotic and proliferative gingival keratinocytes in gingival and sulcular epithelium in patients with gingivitis and periodontitis. J Clin Periodontol 2002; 29:1065-1071.
21. da Costa Filho LC, da Costa CC, Soria ML, Taga R. Effect of home bleaching and smoking on marginal gingival epithelium proliferation: a histologic study in women. J Oral Pathol Med 2002; 31:473-480.
22. Jambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. J Periodontol 1996; 67:1050-1054.
23. Van Winkelhoff AJ, Bosch-Tijhof CJ, Winkel EG, Van der Reijden WA. Smoking affects the subgingival microflora in periodontitis. J Periodontol 2001; 72:666-671.
24. Bostrom L, Linder LE, Bergstrom J. Clinical expression of TNF-alpha in smoking-associated periodontal disease. J Clin Periodontol 1998; 25:767-773.
25. Gultekin SE, Senguven B, Karaduman B. The effect of smoking on epithelium proliferation in healthy and periodontal disease marginal gingival epithelium. J Periodontol 2008; 79:1444-1450.

EFFECTS OF BETEL NUT CHEWING ON LUNG FUNCTION OF STUDENTS IN THE NATIONAL CAPITAL DISTRICT, PAPUA NEW GUINEA

Majella P Norrie, Shalon Taufa and Samson Grant

Discipline of Physiology, Division of Basic Medical Sciences, School of Medicine and Health Sciences, University of Papua New Guinea

Corresponding author: shalontaufa@gmail.com

ABSTRACT:
The effect of betel (areca catechu) nut chewing on the function of the normal human lungs is yet to be fully established. However, it has been noted that chewing betel nut could aggravate acute attacks of asthma in asthmatic patients. A total of 77 students in the age group 19 to 25 years selected randomly in higher learning institutes in Port Moresby Papua New Guinea underwent spirometry tests. Of the 77 students, 34 (44.2%) were males and 43 (55.8%) were females. Out of the 34 male students, 24 (70.6%) were betel nut chewers and 10 (29.4%) were non chewers. Of the 43 female students, 26 (60.5%) were betel nut chewers and 17 (39.5%) were non chewers. There were no statistically significant differences ($p>0.05$) in the spirometry parameters between the chewers and non-chewers in both groups. However, negative correlations were observed between number of nuts chewed per day and FEV_1/FVC in female chewers and also between frequency of chewing and FEV_1 in male chewers.

Keywords: Betel nut, lung function, students, chewers, non-chewers.
Submitted November 2015, accepted December 2015

INTRODUCTION:

Betel nut quid chewing is a common psychoactive substance or masticatory euphoriant used in the Pacific region, South-East Asia, Indo-Pakistan subcontinent, Southern China and coastal areas of East Africa [1, 2]. Betel nut (Areca catechu) chewing is a favourite pastime of Papua New Guineans and is also considered as part of their culture.

Its major alkaloid component, arecoline, is a muscarinic cholinergic agent which acts on the autonomic nervous system and produces various effects like tachycardia, flushing, alertness and warmth [2-5]. It plays a role in the pathophysiology of oral cancers [2], is reported to have cardiovascular effects [6-8] and poor glycemic control [9]. It causes reduction of birth weight among babies of pregnant women who chew betel nut [10].

Its effect on the normal healthy lung is yet to be fully studied. Kiyingi and Taylor noted that healthy subjects who chewed betel nut had no changes in Forced Expiratory Volume in the first second (FEV_1) but a reduced lung function was noted in asthmatic subjects [2,4]. Javed et al found three studies associating areca nut chewing with respiratory discomfort [7].

Although betel nut chewing is very popular in PNG, published data on its effects on the lung function of teenagers and youths is very scanty. The major objective of this study was to assess the pulmonary function of students chewing betel nuts compared to their counterparts that do not chew betel nuts.

SUBJECTS AND METHODS:

Students from three institutions of higher learning in the National Capital District (NCD) of Papua New Guinea (PNG) were recruited to have their pulmonary function tests done in this prospective, observational cross-sectional study. The calculated sample size of 150 was considered appropriate for this study [11].

Students were selected randomly by simple coin toss and then asked to answer a pretested questionnaire. Those who had history of chronic cough, recurrent respiratory tract infection, chest or spinal deformity, asthma, emphysema, Chronic Obstructive Pulmonary Disease (COPD), Tuberculosis (TB) or cardiac illness were excluded from the study [11].

The selected subjects then had their height taken standing erect and upright without shoes and using a standard measuring stadiometer to the nearest millimetre. Weight was also taken without shoes and with light clothing on using a digital weighing scale to the nearest gram. The body mass index was then calculated as appropriate.

Pulmonary function tests were then done with the subject seated comfortably and facing away from the spirometer. The procedure was explained and demonstrated to each student before they proceeded with the test. Subjects used disposable mouthpieces to blow into a computerised spirometer, SpiroUSB model run with Spida5 software [12]. The spirometer was calibrated each morning according to the manufacturer's manual [12].

The best of three good blows was accepted as the final result. Spirometry tests were done using the American Thoracic Society (ATS) guidelines and criteria [11-14].

The pulmonary function tests recorded were: Forced Expiratory Volume in one second (FEV_1), Forced Vital Capacity (FVC), FEV_1/FVC, Peak Expiratory Flow (PEF) and Forced Expiratory Flow 25% and 75% (FEF_{25-75}).

Analysis was done using Microsoft XP Excel data package and SPSS version 20. P-value of <0.05 was considered significant.

Ethical clearance was obtained from the school of medicine and health science ethics committee and each institutional head also agreed to have tests done in their institution. Each of the selected subjects also gave consent before allowed to undergo the tests.

RESULTS:

One hundred and fifty six (156) students volunteered for the test out of which 116 (74.4%) students were randomly selected. Of the 116 students 39 (33.6%) were further excluded after analysis of their questionnaires and their spirometry results. The major reasons for their exclusion were based on the American Thoracic Society (ATS) guidelines and criteria, and also their age [11-14]. Thus, the data obtained from 77 students made up of 34 male (44.2%) and 43 females (55.8%) were finally accepted for analysis. All the students were within the age group 19 to 25 years. Female students had a mean age of 22±1.6 years and male students' 22±1.5 years (Mean ± SD). Out of the 34 male students, 24 (70.6%) were betel nut chewers and 10 (29.4%) were non chewers.

The summary statistics for the pulmonary function indices for male betel nut chewers and non-chewers are shown in Table 1. There were no statistically significant differences ($p>0.05$) in the spirometry parameters of male betel nut chewers compared to their non-betel nut chewing counterparts. Betel nut does not significantly affect the FEV_1, FVC, PEF and FEV_1/FVC% values for male students in the 19 to 25 years age group. Out of the 43 female students, 26 (60.5%) were betel nut chewers and 17 (39.5%) were non chewers. The pulmonary function results for the female chewers and non-chewers of betel nut are shown in Table 2.

No statistically significant differences ($p>0.05$) were obtained in the spirometry results between the female betel nut chewers and the non-chewers.

When the male chewers were compared to the female chewers, males had a significantly higher ($p < 0.05$) mean values for FEV_1, FVC and PEF. However, there was no significant difference ($p>0.05$) between the mean FEV_1/FVC% of the male and female betel nut chewers.

The comparison of the non-betel nut chewers showed that males had a significantly higher ($p=0.001$) FEV_1, FVC and PEF compared to their female counterparts, but there was no significant difference in FEV_1/FVC% between the male and female non-chewers.

The duration of betel nut chewing habits was similar among the male and female chewers; 4 (16.7%) of the male and 4 (15.4%) of the female students had been chewing for less than a year; whereas, 20 (83.3%) of the male and 22 (84.6%) of the female students had been chewing for over a year.

The number of betel nuts chewed per day was also similar among the male and female students.

Among the 24 male chewers, 19 (79.2%) chewed one to two betel nuts per day compared to 20 (76.9%) of the 26 female chewers. More than 3 betel nuts per day were chewed by 5 (20.8%) male students compared to 6 (23.1%) female students.

Table 1: Summary statistics of the Pulmonary Function Indices for male betel nut chewers and non-chewers

Parameters	FEV_1 (Litres)		FVC (Litres)		PEF (Litres/min)		FEV_1/FVC (%)	
	Chewers	Non chewers	Chewers	Non chewers	Chewers	Non chewers	Chewers	Non chewers
Mean	3.68	3.76	4.15	4.23	601.0	581.1	88.9	88.9
Std. Dev	0.43	0.42	0.57	0.50	83.3	135.7	4.4	3.9
Range	2.87- 4.51	2.96- 4.38	3.23- 5.52	3.43- 4.95	460.0 - 785.0	415.0-767.0	79.0 - 96.0	81.0 - 95.0
95% CI	3.49- 3.86	3.46- 4.06	3.91- 4.39	3.87- 4.58	565.9 - 636.2	484.0-678.2	87.0 - 90.7	86.1- 91.7
Median	3.67	3.84	4.03	4.28	601.5	552.0	89.0	89.0
Interquartal Range (IQR)	3.42- 3.96	3.51- 4.02	3.73- 4.51	3.78- 4.67	555.2 - 640.5	451.5-743.8	86.3 - 92.0	87.5 - 91.0
Mann Whitney (p)	0.515		0.707		0.467		0.985	

Table 2: Summary statistics of the Pulmonary Function Indices for female betel nut chewers and non-chewers

Parameters	FEV_1 (Litres)		FVC (Litres)		PEF (Litres/min)		FEV_1/FVC (%)	
	Chewers	Non chewers	Chewers	Non chewers	Chewers	Non chewers	Chewers	Non chewers
Mean	2.97	2.82	3.34	3.11	466.3	434.8	88.9	90.8
Std. Dev	0.42	0.32	0.60	0.38	74.8	65.3	4.4	3.6
Range	2.39-3.79	2.35-3.49	2.64-4.81	2.56-3.95	359.0-655.0	326.0-569.0	79.0-96.0	85.0-98.0
95% CI	2.80-3.14	2.66-2.99	3.10-3.58	2.92-3.30	436.1-496.5	401.2-468.3	87.0-90.7	88.9-92.6
Median	2.92	2.79	3.16	3.10	471.0	430.0	89.0	91.0
IQR	2.64-3.31	2.59-3.06	2.85-3.80	2.89-3.37	402.5-508.0	385.0-489.5	86.3-92.0	88.0-92.5
Mann Whitney (p)	0.308		0.378		0.180		0.600	

Results presented in table 3 show the data obtained for the frequency of chewing by the male and female students. The majority, 41.7% of male and 50% of female students have been chewing betel nut every day since they started chewing.

Tables 4 and 5 show the correlation of duration, frequency and number of nuts chewed per day with pulmonary function among the female and male chewers respectively.

There is an inverse statistically significant relationship between the number of nuts chewed per day and the FEV_1/FVC ratio (rho= -0.471, p=0.015) among the female chewers.

Among the male chewers correlation coefficient showed inverse statistically significant relationship between the frequency of chewing betel nut and the FEV_1. (rho= -0.504, p=0.012).

Table 3: Frequency of betel nut chewing by the male and female chewers

Frequency	Male students (n=24)	Female students (n=26)
Every day	10 (41.7%)	13 (50.0%)
Every other day	6 (25.0%)	2 (7.7%)
Once/week	6 (25.0%)	6 (23.1%)
Once/month	0	4 (15.4%)
Once or twice a year	2 (8.3%)	1 (3.8%)

Table 4: Correlation between the duration, frequency of chewing and number of nuts chewed per day, and the pulmonary function of female chewers

	FEV_1		FVC		FEV_1/FVC	
	r*	p-value	r*	p-value	r*	p-value
Duration	-0.128	0.533	-0.299	0.138	0.157	0.443
Frequency	0.131	0.522	0.023	0.910	0.059	0.774
Number of nuts/day	0.095	0.645	0.243	0.232	**-0.471**	**0.015**

*Spearman correlation coefficient; p-value <0.05 is significant.

Table 5: Correlation between the duration, frequency of chewing and number of nuts chewed per day, and the pulmonary function of male chewers

	FEV_1		FVC		FEV_1/FVC	
	r*	p-value	r*	p-value	r*	p-value
Duration	0.307	0.145	0.097	0.652	0.235	0.269
Frequency	**-0.504**	**0.012**	-0.337	0.107	0.213	0.318
Number of nuts/day	0.332	0.113	0.034	0.875	0.106	0.621

*Spearman correlation coefficient; p-value <0.05 is significant.

DISCUSSION:

Betel nut chewing is a habit prevalent in PNG. Betelnut causes bronchoconstriction in asthmatic patients and thus aggravates their asthma [2, 4]. Kiyingi and Saweri looked at the effects of betelnut in healthy non-asthmatic subjects and found no changes in FEV_1 [2]. Taylor and colleagues also tested arecholine inhalation in healthy subjects. Even though there was more bronchoconstriction found in asthmatic people (6 out of 7 patients), one out of six healthy subjects developed bronchoconstriction [4]. Yanga and Datta studied Melanesian male 18-40 year olds and found that betel nut chewers had significantly reduced FVC and FEV_1 [3].

Our results, however, showed no significant difference between male and female chewers and non-chewers of betel nut. Yanga and Datta [3] excluded smokers from the chewing and non-chewing groups and only included chronic chewers (those who chewed 3-5 betel nuts per day for 2-5 years).

In this study chewers could also be smokers and the definition of chewing encompassed all types of chewers. This could explain why we didn't identify any significant differences between the two categories.

In a recent study published in 2014 by Wang et al, a large sample of 600 asthma patients and 1200 controls were studied to investigate the connection between asthma and betel nut use [15]. They found that a higher arecoline level was associated with worse lung function FEV_1 (rho = -0.359, p=0.004), FVC (rho = -0.309, p=0.02) in the male asthma group and concluded that betel nut chewing is associated with asthma. Correlation coefficient of the case group was not done.

Our study, however, looked only at apparently healthy young people. The number of betel nuts chewed per day by the female chewers was negatively correlated with FEV_1/FVC and the frequency of chewing was negatively correlated with FEV_1 among the male chewers. A suggested larger sample size may identify more significant changes.

CONCLUSION:

This study shows negative correlations between betel nut chewing and lung function of students aged 19 to 25 years in Port Moresby Papua New Guinea.

ACKNOWLEDGEMENTS:

We thank the School of Medicine & Health Sciences University of Papua New Guinea for the research grant used in this project. We are grateful to all the students in Port Moresby Business College, Don Bosco Technological Institute and the University of Papua New Guinea for their participation in this study.

REFERENCES:

1. Gupta, P.C. Epidemiology of betel quid usage. Ann Acad Med Singapore 2004; 33 (suppl): 31s-36s.

2. Kiyingi, S.K and Saweri, A. Betel nut chewing causes bronchoconstriction in some asthma patients. PNG Med J 1994: 37(2): 90-99.
3. Datta, S and Yanga, J.K. Comparison of the Effects of Chronic Smoking and Betel Nut Chewing on the Respiratory and Cardiovascular Parameters in Melanesian Male Population. Medical Sciences Bulletin 2003; 1: 13-17.
4. Taylor, R.F.H, Al-Jarad, N, John, L.M.E and Barnes, N.C. Betel nut chewing and asthma. The Lancet 1992; 339: 1134-1136.
5. Deng, JF, Ger, J, Tsai, WJ, Kao, WF, Yang, CC. Acute Toxicities of Betel Nut: Rare but probably overlooked events. Journal of Toxicology: Clinical Toxicology. 2001; 39(4): 355-60.
6. Kevau I.H, Miam B, Jothimanikam J, Urae G, Itaki R, Mari R, Wagiebu J, Sengupta A. Betel nut causes paradoxical vasoconstriction in patients with coronary artery disease – an exciting new discovery in Papua New Guinea with important clinical implications. Cardiac Society of Australia and New Zealand, 47th Conference presentation, 1998.
7. Javed, F, Fernando, O, Correa, B, Chotai, M, Tappuni, AR, Almas, K. Systemic conditions associated with areca nut usage: A Literature Review. Scandinavian Journal of Public Health. Dec, 2010; 38(8): 838-44. doi: 10.1177/1403494810379291
8. McClintock, T.R, Parvez, F, Wu, F, Wang, W, Islam, T, Ahmed, A, Shaheen, I, Sarwar, G, Demmer, R.T, Desvarieux, M, Ahsan, H, Chen, Y. Association between betel quid chewing and carotid intima – media thickness in rural Bangladesh. Int J Epidemiol 2014; 43(4): 1174-1182.
9. Amos L.B, Margis D. Betel nut chewing: a contributing factor to the poor glycaemic control in diabetic patients attending Port Moresby General Hospital, Papua New Guinea. PNG Med J 2005; 48(3-4): 174-182.
10. Senn,M, Baiwog,F, Winmai, J, Mueller I, Rogerson, S, Senn,N. Betel Nut chewing during pregnancy, Madang Province, Papua New Guinea. Drug and Alcohol Dependence Nov 2009; 1105(2): 126-131.
11. Norrie, M.P, Taufa S. Assessment of Pulmonary Function in Healthy Students aged 19 to 25 years in the National Capital District, Papua New Guinea. Pac J Med Sci 2013; 12(1): 55-65.
12. Micro Medical Limited. SpiroUSB Operating Manual. 2004 [Available at www.diagnostikk.no/33/Spiro_USB_operating_manualengelsk.pdf:(accessed April 2014)]
13. American Thoracic Society. Lung function testing: selection of reference values interpretative strategies. Am Rev Respir Dis 1991; 144(5): 1202-18.
14. Miller, M.R, Hankinson, J, Brusasco, V, Burgos, F, Casaburi, R, Coates, A, et al. Standardisation of Spirometry. Eur Respir J 2005; 26: 319-338.
15. Wang, T.N, Huang, M.S, Lin, M.C, Duh, T.H, Lee, C.H, Wang, C.C, Chen, P.H, Chiang, S.L, Sheu, C.C, Chen, V.C.H, Wu, C.C, Ferri, C.P, Stewart, R, Ko, Y.C. Betel chewing and Arecoline affects Eotaxin-1, asthma and lung function. PLOS ONE, March 2014, 9(3); 1-8.

DRUG SUSCEPTIBILITY PATTERN OF MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM PATIENTS UNDERGOING FINE NEEDLE ASPIRATION BIOPSY AT PORT MORESBY GENERAL HOSPITAL, PAPUA NEW GUINEA

***Rodney Itaki, **Francis Bannick, ***Evelyn Lavu, **Jacklyn Joseph, ***Ruth Magaye, ***Jennifer Banamu, ***Karen Johnson, ^Henry Welch**

***Division of Pathology and ^Division of Clinical Sciences, School of Medicine and Health Sciences University of Papua New Guinea; **Pathology Department Port Moresby General Hospital; ***Central Public Health Laboratories, National Department of Health Papua New Guinea;**

Corresponding author: ritaki@upng.ac.pg

ABSTRACT:
Drug resistant TB is increasing in Papua New Guinea. Although drug resistant data of mycobacterial isolates from pulmonary TB cases is available, there is limited information on culture confirmed drug resistant tuberculous lymphadenitis. In the framework of a pilot study evaluating the use of Xpert MTB/RIF in diagnosing tuberculous lymphadenitis compared to microscopy at the Port Moresby General Hospital, 18 fine needle aspiration biopsy samples were sent to Brisbane, Australia for culture. We report the drug susceptibility testing results of 12 isolates (12/18). The mycobaceterial yield was 66.7% (12/18) with 58.3% (7/12) of the isolates showing drug resistance to at least one drug tested. Mono-resistant rate was 25% (3/12) whereas Multi Drug Resistant TB (MDR-TB) rate was 33.3% (4/12).

Keywords: fine needle aspiration biopsy, tuberculous lymphadenitis, GeneXpert, Xpert MTB/RIF, drug susceptibility testing, Papua New Guinea
Submitted: November 2015, Accepted: December 2015

INTRODUCTION:

The World Health Organisation (WHO) estimates that about 8 million people get infected with mycobacterium tuberculosis (MTB) each year of which nearly two million die [1]. In Papua New Guinea (PNG) the estimated incidence of tuberculosis (TB) is 532 per 100 000 per annum [2,3]. Drug resistant TB and multi-drug resistance-TB (MDR-TB) are major challenges in the fight against TB in PNG [3]. The recently completed PNG TB drug resistant survey is expected to provide accurate data on drug resistant pattern of MTB isolates in PNG [personal communication, Lavu 2015]. PNG does not have drug susceptibility testing (DST) facilities and samples that require DST are sent to the Queensland Mycobacterium Reference Laboratory (QMRL) in Brisbane, Australia. There are plans to re-establish MTB culture facilities at the PNG Central Public Health Laboratories

(CPHL) and it is expected to be operational in 2016. A review of the DST patterns of MTB isolates from the Western Province of PNG showed isolates to be resistant to at least one of the five primary drugs used for treating TB [2]. The rate of MDR-TB in that study was 26% [2].

Ley et al examined DST patterns of isolates from Goroka, Madang and Alotau that showed 10.8% of isolates were resistant to at least one drug tested where 30.4% were mono-resistant to Streptomycin, 17.4% to Isoniazid and 13.0% mono-resistant to Rifampicin [4]. In that study, isolates from Alotau had a MDR-TB rate of 4.6% [4]. These aforementioned DST patterns are from isolates obtained from sputum samples. A study in Ethiopia showed 6.7% of MTB isolates from lymph node aspirates were resistant to at least one first line anti-TB drug [1]. In that same study the MDR-TB rate was 1.3% [1]. There is limited data on the DST pattern of isolates obtained from patients with tuberculous lymphadenitis (TBLN) in PNG. We report the DST pattern of 12 MTB isolates cultured from fine needle aspiration biopsy (FNAB) aspirates at the Port Moresby General Hospital (PMGH).

METHODS:

Study design and sampling

In the framework of a prospective descriptive pilot study comparing Xpert MTB/RIF (Xpert) and microscopy in diagnosing TB lymph node at PMGH, FNAB samples requiring DST were sent to Australia for culture. Every third consecutive patient was chosen and interviewed. Study subject were recruited by purposive convenience sampling. A total of 107 study participants were recruited from the weekly FNAB clinic at PMGH. Informed written consent was obtained from all study participants. Parents and guardians of children aged 13 years and below gave written and informed consent prior to recruitment into the study. In-patients requiring FNAB for diagnostic workup were also included. Study participants were allowed to withdraw from the study at any time. Basic demographic and clinic data were obtained using a pre-tested questionnaire. Patients with breast lumps and thyroid enlargement presenting for FNAB were excluded from the study. Ethical approval for the study was obtained from the PNG National Department of Health Medical Research Advisory Committee (MRAC File Number 54-6-2).

Fine needle aspiration and laboratory testing procedure:

A 23 gauge hypodermal sterile needle was attached to a 10ml syringe and the FNAB samples were obtained without a syringe pistol. If there were multiple glands, samples were obtained from the largest gland. Samples were obtained from cervical, submandibular, axillary and inguinal lymph nodes. After cleaning the skin with 70% alcohol skin swab, while using the non-dominant hand to fix the enlarged gland and immobilising it, the dominant hand holding the syringe with attached needle was inserted into

the gland. A suction pressure of 2.0ml was applied and sample obtained by moving the needle back and forth without completely withdrawing the needle. Suction was stopped and needle withdrawn when sample was visible at the hub of the needle. Maximum of two passes were done if first obtained insufficient material. Standard smears were then made on frosted glass slides for Ziehl-Neelsen and modified Wright Giemsa staining. The remainder of the FNAB specimen in the needle hub was dispensed into a sterile container with 2ml physiological saline for Xpert testing at the CPHL. Physiological saline has been shown to be a good transport and storage medium of FNAB aspirates for Xpert analysis [4].

The WHO Xpert implementation manual and CPHL standard operating procedures were used as guide for Xpert result interpretation [6]. Specimen preparation for Xpert analysis was modified from Malbruny et al [5]. Modification was as follows:

- 1.0ml of the aspirate-saline mixture was transferred into another sterile container of 1.0ml physiological saline to make it up to 2.0ml. This was done to allow better emulsification of the mucoid or blood stained material by the Xpert sample preparation buffer and to allow enough volume to work with.
- The Xpert sample preparation buffer was added to the mixture at a ratio of 2:1.
- After votexing, the mixture was incubated at room temperature for 10 minutes. The mixture was vortexed again and incubated at room temperature for a further 5 minutes.
- 2.0ml of the mixture was dispensed into the Xpert cartridge and processed using the manufacturer's protocol (Cepheid, USA).

The remainder of the unprocessed aspirate-saline sample following Xpert testing was sent to the QMRL in Australia for DST if rifampicin resistance was detected by Xpert. The QMRL is the usual laboratory for MTB DST on samples from PNG [7]. Criteria for culture were (1) Rifampicin resistance by Xpert and (2) as a component of the quality assurance program at CPHL. Personal protection equipment was worn and standard biosafety procedures were followed at all times during the sampling and testing processes.

RESULTS:

A total of 107 patients were recruited for the study and all of them consented to participate. Thus the consent rate was 100%. However, culture was done on the sample from 18 (16.8%) patients. Mycobacterium tuberculosis complex was isolated from 12 of the 18 (66.7%) samples. The mean (± Std Dev) age of the 12 patients was 24.3 ± 8.5 years, median age 24.5 years and age range was 11.0 to 40.0 years.

Table 1: Basic demographic and clinical data of positive culture

Isolate No.	Age	Gender	Aspirate type	Positive family history	Previously treated for TB	Place of residence	MTB detected by Xpert	Rifampicin resistance detected by Xpert	Microscopy result	Resistance detected By culture
1	23	F	blood+pus	No	Yes	Rural	Yes	No	TB	Nil
2	30	F	blood+pus	No	No	Settlement	Yes	Yes	TB	Nil
3	31	F	pus	No	No	Urban	Yes	Yes	TB	RIF
4	12	M	pus	No	Yes	Settlement	Yes	Yes	TB	RIF
5	32	M	blood	No	No	Rural	Yes	Yes	TB	Nil
6	26	F	blood	No	No	Urban	Yes	Yes	TB	Nil
7	26	M	blood+pus	No	No	Urban	Yes	Yes	TB	Nil
8	11	M	blood+pus	Yes	Yes	Peri-urban village	Yes	Yes	Suppurative lymphadenitis	RIF, INH, EMB, PZA, ETH
9	23	M	pus	No	No	Urban	Yes	Yes	TB	RIF, INH, ETH
10	40	M	blood+pus	Yes	Yes	Rural	Yes	Yes	TB	RIF
11	22	M	pus	yes	Yes	Settlement	Yes	Yes	Suppurative lymphadenitis	RIF, INH, ETH
12	16	M	blood+pus	No	No	Settlement	Yes	Yes	TB	RIF, INH, ETH, O

RIF= Rifampicin, INH = Isoniazid, ETH = Ethionamide, O = Ofloxacin, PZA = Pyrazinamide, EMB = Ethambutol.

Table 2:.Basic demographic and clinical data of negative culture

Isolate No.	Age (years)	Gender	Aspirate type	Positive History	Previously treated for TB	Place of residence	MTB detected by Xpert	Rifampicin resistance detected by Xpert	Microscopy result	culture
1	1.9	M	Blood	Yes	No	Rural	No	-	Non-specific lymphadenitis	No growth
2	28	F	Blood+pus	Yes	No	Urban	Yes	Yes	TB	No growth
3	21	F	Blood+pus	Yes	No	Urban	Yes	Yes	TB	No growth
4	26	F	Blood+pus	No	Yes	Urban	Yes	No	TB	No growth
5	40	F	Blood+pus	Yes	No	Settlement	Yes	Yes	Suppurative lymphadenitis	No growth
6	52	M	Pus	Yes	Yes	Urban	Yes	No	TB	No growth

Gender distribution of the 12 patients indicated 8 (66.7%) males and 4 (33.3%) females. The mean, median and age range of the male patients were 22.8 ± 10.0 years, 22.5 years and 11.0 to 40.0 years respectively. For the female patients the corresponding values were 27.5 ± 3.7 years, 28.0 years and 23.0 to 31.0 years respectively.

Of the 12 isolates there were seven (58.3%) resistant isolates four (33.3%) of which were MDR-TB. Three patients (25%) had positive family history and 5 (41.7%) were previously treated for TB. Of the four patients with MDR-TB, only one (25.0%) had a history of previously being treated for TB. Four patients (33.3%) were from settlements, 3 (25%) from rural villages outside Port Moresby, 1 (8.3%) from a peri-urban village and 4 (33.3%) were from urban municipal residential areas in Port Moresby. Xpert detected MTB in 17 samples (94.4%) whereas microscopy was negative in 4 (22.2%). Two (50%) of the negative microscopy specimen grew MTB complex. Table 1 shows the basic demographic and clinical data of the 12 patients with positive isolates. Table 2 shows the clinical and demographic data of the six patients with culture negative samples.

DISCUSSION:

Three categories of drugs are available for use in PNG to treat TB [3]. The drugs are available for use as fixed dose combinations and are used for PTB and EPTB treatment. Category one are used for all new TB cases, category two for all re-retreatment cases and drug resistant cases are treated with category four [3]. Category one drugs includes rifampicin, isoniazid, pyrazinamide and ethambutol. Category two drugs includes all the category one drugs plus streptomycin and category four drugs are pyrazinamide, kanamycin, levofloxacin, ethionamide, cycloserine, capreomycin and para-amniosalicylic acid. MTB isolates from Western Province were shown to be resistant to all category one and two drugs except amikacin and kanamycin [2]. Isolates from Goroka, Madang and Alotau have been found to have mono-resistant rates of 8.9%, 4.6% and 6.7% respectively [4]. In our cohort of 12 isolates the mono-resistant rate was 25% (3/12). Whereas isolates from Goroka, Madang and Alotau showed mono-resistance to streptomycin and isonidazid, no isolates in our study showed resistance to these drugs. However, our results are from 12 isolates whereas Let et al analysed more than 50 isolates from each town [4].

Compared to other studies on MDR-TB in PNG our cohort had a MDR-TB rate of 33.3% (4/12).This high rate obtained in a pilot study but indicates the need for a larger sample size study at PMGH. Two of MDR-TB cases were from settlements, one from a peri-urban village and one was from a village outside Port Moresby. Two patients with MDR-TB had neither past history of being treated for TB nor positive family history. Genetic analysis of drug resistant MTB

isolates from PNG has shown identical resistance-conferring mutations within clustered isolates suggesting patient to patient transmission of drug resistant TB [7]. We did not conduct genetic analysis on the MTB isolates but the results indicate a need for molecular epidemiological studies using MTB isolates from patients at PMGH. The MDR-TB isolates showed resistant to rifampicin, ethambutol, ethionamide and pyrazinamide. This pattern is similar to other published data on TB drug resistance in PNG [2,7,4,]. Isolates in our study did not show resistance to streptomycin, capreomycin, cycloserine and para-aminosalicylic acid (PAS) which is different to other studies in PNG [2,4]. A study in Ethiopia suggests newly identified strains of MTB may have an affinity for lymph nodes causing tuberculous lymphadenitis [10]. It is not known if these new strains have a different DST pattern compared to isolates from patients with PTB.

Two positive cultures were from children of which one was a MDR-TB isolate. Obtaining positive culture in children is very challenging and using a modified sample processing procedure, MTB complex was isolated. The procedure described can be used to investigate children with TBLN that are not responding to first line TB drugs. The procedure is also useful to sample enlarged lymph nodes for Xpert analysis. Fine needle aspiration biopsy of enlarged node is cheap and easy to perform with minimal training and other health care workers can be trained to perform this procedure at the outpatient enabling sample collection for culture [11]. Availability of Xpert in 21 hospitals in PNG further offers a means of laboratory confirmation of TBLN. Readily available infrastructure and equipment for laboratory confirmation of TBLN will ensure rapid case detection and treatment of cases. Further, culture confirmation and rapid dissemination of DST results will help combat drug resistant TB in PNG.

This study had a mycobacterial culture yield of 66.7% (12/18). Culture yield of MTB from FNAB is reported to be between 42% and 83% [12]. Positive human immunodeficiency virus infection (HIV) is also associated with a higher yield from FNAB aspirates in adults [12]. We did not investigate for HIV status due ethical considerations in the study design. Factors relating to the negative culture may have included inadequate specimen (1 patient) or TB treatment for more than 2 weeks (2 patients). Although we used physiological saline for emulsifying the FNAB aspirate for culture, the yield is higher in MTB-specific transport mediums [12]. Prolonged storage of specimen prior to shipment to Australia may have also resulted in the negative cultures.

CONCLUSION:

MTB can be cultured from FNAB aspirates emulsified in physiological saline. The DST pattern of the 12 isolates from FNAB aspirates in this study suggests the possibility of a difference

in DST pattern between PTB and TBLN at PMGH. However, a larger sample size is needed to confirm these possible differences.

REFERENCE:

1. Biadglegne F, Tessema B, Sack U, Rodloff A.C. Drug resistance of Mycobacterium tuberculosis isolates from tuberculosis lymphadenitis patients in Ethiopia. The Indian journal of medical research 2014;140:116-122.
2. Simpson G, Coulter C, Weston J, Knight T, Carter R, Vincent S, Robertus L, and Konstantinos A. Resistance patterns of multidrug-resistant tuberculosis in Western Province, Papua New Guinea [Notes from the field].The International J of Tuberculosis and Lung Disease 2011;4(15):551-552.
3. Papua New Guinea National Tuberculosis Management Protocol. Papua New Guinea National Department of Health 2013.
4. Ley S, Harino P, Vanuga K, Kamus R, Carter R, Coulter C, Pandey S, Feldmann J, Balif M, Siba P, Phuanukoonnon S, Gagneux S, Beck H. Diversity of Mycobacterium tuberculosis and drug resistance in different provinces of Papua New Guinea.BMC microbiology 2014;14:307.
5. Malbruny B, Marrec G.L, Courageous R, Leclercq R, Cattoir L.V. Rapid and efficient detection of Mycobacterium tuberculosis in respiratory and non-respiratory samples. Int J Tuberc Lung Dis. 2011;15(4):553-555.
6. World Health Organisation. Xpert MTB/RIF implementation manual. Technical and operational 'how to' practical considerations 2014.
7. Ballif M, Harino P, Ley S, Coscolla M, Niemann S, Carter R, Coulter C, Borrell S, Siba P, Phuanukoonnon S, Gagneux S, Beck H. Drug resistance-conferring mutations in Mycobacterium tuberculosis from Madang, Papua New Guinea. BMC microbiology 2012;12:191.
8. Cross G.B, Coles K, Nikpour M, Moore O.A, Denholm J, McBryde E.S, Eisen D.P, Warigi B, Carter B, Pandey S, Harino P, Siba P, Coulter C, Mueller I, Phuanukoonnon S, Pellegrini M. TB incidence and characteristics in the remote gulf province of Papua New Guinea: a prospective study. BMC infectious diseases 2014;14:93.
9. Gilpin C.M, Simpson G, Vincent S, O'Brien T.P, Knight T.A, Globan M, Coulter C, Konstantinos A. Evidence of primary transmission of multidrug-resistant tuberculosis in the Western Province of Papua New Guinea.Medical Journal of Australia 2008;3(188):148-152.
10. Biadglegne F, Merker M, Sack U, Rodloff A.C, Niemann s. Tuberculous Lymphadenitis in Ethiopia Predominantly Caused by Strains Belonging to the Delhi/CAS Lineage and Newly Identified Ethiopian Clades of the Mycobacterium tuberculosis Complex. PloS One 2015;9(10):e0137865.
11. Ligthelm LJ, Nicol MP, Hoek KGP, Jacobson R, van Helden PD, Marais BJ, Waren RM, Wright CA. Xpert MTB/RIF for rapid diagnosis of tuberculous lymphadenitis from fine-needle-aspiration biopsy specimens. Journal of clinical microbiology 2011;49 (11):3967-3970.
12. Razack R, Louw M, Wright C.A. Diagnostic yield of fine needle aspiration biopsy in HIV infected adults with suspected mycobacterial lymphadenitis S Afr Med J 2014;104(1):27-27. DOI:10.71956/SAMJ.7492.

EFFICACY AND TOLERABILITY OF FIXED DOSE COMBINATION OF SILYMARIN, ALPHA LIPOIC ACID, N-ACETYL CYSTEINE AND SELENIUM IN THE MANAGEMENT OF SOME LIVER DISORDERS

***^ARIF A. FARUQUI, **SHASHIKANT PAWAR, ***SANJOY DASGUPTA**

*Clinical Pharmacologist, Mumbai Maharashtra, India; **Pawar Nursing Home, Satara, Maharashtra, India
***Gangulibagan, Jadavpur, Kolkata-700047, West Bengal, India

^Corresponding Author: drfaruqui@gmail.com

ABSTRACT:
Globally liver disorders are major cause of illness death and death. Oxidative stress plays a critical role in the progression of alcoholic and nonalcoholic related diseases. The aim of the present study was to evaluate the efficacy and tolerability of fixed dose combination (FDC) of silymarin, alpha lipoic acid, N-acetyl cysteine and selenium in the management of liver disorders. This was an observational, non-randomized, open label, non-comparative, multi-centric post-marketing surveillance study. The above mentioned FDC was administered to 15 patients diagnosed with alcoholic or viral hepatitis for three months. Evaluation of liver function tests (LFT) were carried out at baseline and at the end of 3rd month of the treatment. Significant changes were observed in the LFT parameters at the end of three months of this study. aspartate aminotransferase (AST): (Mean ± SEM) 369.9 ± 128.0 to 97.00 ± 34.27 U/L, ($p < 0.0001$); alanine aminotransferase (ALT): 652.93 ± 214.57 to 194.40 ± 82.51 U/L, ($p < 0.03$); Alkaline phosphatase: 197.47 ± 25.57 to 151.60 ± 17.92 U/L, ($p < 0.0059$); Gamma glutamyl transferase: 156.67 ± 49.80 to 87.33 ± 22.94 U/L, ($p < 0.0490$); Total bilirubin: 3.44 ± 0.76 to 1.66 ± 0.57 mg/dL, ($p < 0.0192$) and bilirubin direct: 2.13 ± 0.58 to 1.00 ± 0.50 mg/dL, ($p < 0.0273$). Two patients reported mild gastrointestinal adverse events (nausea, bloating). This FDC was therapeutically effective under the circumstances of elevated oxidative stress and produces significant reduction in LFT parameters in alcoholic and viral hepatitis patients.

Keywords: Alcoholic liver disease, Oxidative Stress, Silymarin, Alpha lipoic acid, N-acetyl cysteine, Selenium
Submitted November 2015, Accepted December 2015

INTRODUCTION:

Liver disorders are the major cause of illness and death worldwide [1]. Although liver disease is stereotypically linked to alcohol or drugs, the truth is that there are more than 100 known forms of liver disease caused by various factors and affecting all age groups [2]. The major factors thus include viruses, alcohol, obesity and drugs. Overall half of global population is exposed to different forms of hepatotrophic viruses, suggesting viral hepatitis as a commonest cause of acute and chronic liver disorders [3]. In India,

40 million people are infected with hepatitis B, constituting approximately 11 per cent of the estimated global burden [4].

Alcoholic liver disease, another major liver disorder involves liver damage due to alcohol abuse and usually occurs after several years of excessive alcohol consumption. Pathological steps in deterioration of liver function involve steatosis, steato-hepatitis to fibrosis and cirrhosis [5]. Furthermore in liver cirrhosis, it was established that chronic alcohol consumption is a risk factor for the development of hepatocellular carcinoma [6]. Moreover in the past 30 years, deaths due to liver cirrhosis have steadily increased with values more than 1 million in the year 2010 making it 2% of all deaths in that year. In Asia, hepatitis B and hepatitis C accounts for more than half of the liver cirrhosis burden [7]. Liver disease related to ethanol is a common problem and is one of the major medical complications of alcohol abuse. Daily consumption is usual for causation of liver disease. The World Health Organization (WHO) estimates that 140 million people worldwide suffer from alcohol dependency that produces damage to lives and economies [8].

Oxidative stress is defined as "a disturbance in the pro-oxidant-antioxidant balance in favor of the former". Oxidative stress plays a critical role in the progression of alcohol related liver disease [9]. Alcohol and its immediate metabolite acetaldehyde increases liver oxidative stress through generation of highly reactive oxygen species (ROS), reactive nitrogen species (RNS) and adducts that can injure the hepatocytes and liver parenchyma [10]. Apart from generating these free radicals, chronic alcohol ingestion significantly affects hepatocytes by depleting important components of antioxidant defense system (enzymatic and non-enzymatic). This imbalance created by alcohol mediated elevated generation of pro-oxidants as well as depletion in the enzymatic and non-enzymatic antioxidant defense systems in liver may contribute to the progression and development of alcoholic liver disease [11].

Prevention of ROS mediated damage is accomplished with the help of enzymatic and nonenzymatic processes. Glutathione peroxidase, Catalase and Superoxide dismutase (SOD) are supposed to be the primary antioxidant enzymes, as their involvement leads to direct elimination of ROS. Glutathione peroxidase, a selenoprotein, is a cytoplasmic and mitochondrial enzyme, crucial for detoxification of hydrogen peroxide in almost all the cells. Glutathione peroxidase contains a seleno-cysteine amino acid at the active site instead of a normal cysteine. Another important component of antioxidant network is Reduced Glutathione (GSH) which protects the human body from free radicals. Glutathione exists in the metabolic system as either in Reduced (GSH) or oxidized (GSSG) states. In reduced state, glutathione can donate an electron and stabilize a free radical. During this donation the glutathione becomes highly reactive and reacts with another glutathione molecule to form oxidised glutathione (GSSG). The GSSG is then converted to

Reduced Glutathione (GSH) by the enzyme glutathione reductase [11].

Critical biochemical manifestation of alcoholic and nonalcoholic liver diseases is thus sustained increased oxidative stress. Alcohol, its metabolites and viral infections tip this balance in favour of oxidative stress. It is therefore, worthwhile to estimate the extent of oxidative stress and the front line component of antioxidant defense system in these patients. Current research plays an important role in recognizing the role of oxidative stress with respect to disease severity to build up the antioxidant defense system in the supportive management of oxidative stress induced liver disorders. Administration of antioxidant is therefore a vital therapeutic strategy and advocates that routine screening of antioxidant levels should be done to find out the deficient status [11].

Medicinal agents with antioxidant potential include not only vitamin A, C, and E, but also complementary and alternative medicine agents such as silymarin, S-adenosylmethionine, alpha lipoic acid, N-acetyl cysteine (NAC) and trace elements, such as zinc and selenium. Although most of these agents have numerous effects, such as free radical scavenging, membrane-stabilizing and anti-inflammatory properties, most experimental data reinforces ability of these agents in lowering oxidative stress as a fundamental of their beneficial effect [12]. In India and across the world, patients with chronic liver disorders are known to use these complementary and alternative medications; one study in the United States of America revealed that 39% of patients in their study used these agents, and another such study in Germany reported 65% of patients. This number probably underestimates usage, because many surveys show that 31%-40% of patients do not disclose their use of above mentioned agents to their physicians [12].

Silymarin, conventionally known as 'milk thistle' is one of the oldest and intensively researched medicinal plant in the treatment of liver diseases. Traditionally, milk thistle has been used for alcoholic hepatitis, alcoholic fatty liver, liver poisoning, ischemic injury, liver cirrhosis and viral hepatitis. Silymarin has been proclaimed to safeguard the hepatocytes from wide range of toxins, including ethanol, acetaminophen, carbon tetrachloride, arsenic and radiation [13]. N-acetyl-cysteine (NAC) is another vital antioxidant and is quite popular for its capacity to minimize and downstream the negative effects associated with oxidative stress. NAC is largely acknowledged to minimize the lipid peroxidation of cellular membranes and other such targets that is known to occur with oxidative stress [14]. Alpha-lipoic acid is manufactured in almost all tissues and is competent in solubilising in both water and fats thus can be useful throughout the body. Physiologically as an antioxidant, alpha-lipoic acid directly terminates free radicals, chelates metal ions, increases levels of cytosolic glutathione and vitamin C [11].

Selenium is a non-metal that exists in several oxidation states and is an essential component of the glutathione peroxidase enzyme system with a significant function of protecting the cell from the oxidative stress and free radical formation. The

"rate limiting" substrate in the GSH-GSSG oxido-reduction is selenium and its deficiency affects the generation of peroxidase enzyme leading to deterioration of the antioxidant protection by severely reducing the GSH-GSSG levels. Selenium-deficiency has been also shown to result in less robust immune responses to viruses, tumors, and allergens, in comparison to selenium-adequate control population. Selenium deficiency has been implicated as a cause of hepatic injury, possibly from accentuated lipoperoxidation due to diminished activity of the selenoenzyme, glutathione peroxidase, despite the absence of severe malnutrition [15]. Clinical studies have also confirmed that, in patients with alcoholic and nonalcoholic liver diseases, hepatic selenium levels are reduced compared to healthy population, suggesting the positive role of selenium supplementation in liver disorders [16]. Selenium is a potent nutritional antioxidant that carries out biological effects through its incorporation into seleno-proteins. Selenium is an active immune-modulator, much more potent anti-oxidant than vitamins E, C and A, beta-carotene [17, 18].

In light of the vital role of ROS and oxidative stress in the pathophysiology of liver diseases, antioxidants are understandably considered as a crucial therapeutic approach for the management of liver disorders [19]. Therefore, this study was designed to evaluate the efficacy and tolerability of fixed dose combination (FDC) of silymarin, alpha lipoic acid, N-acetyl cysteine and selenium in the management of patients suffering from alcoholic and nonalcoholic liver disorders.

METHODS:

This was an observational, non-randomized, open label, non-comparative, multi-centric post-marketing surveillance study. The FDC of silymarin, alpha lipoic acid, N-acetyl cysteine and selenium was orally administered as twice daily to the patients suffering from liver disorders including alcoholic and viral hepatitis for at least 3 months. Clinical diagnosis was performed based on patients clinical and biochemical investigations conducted at baseline. Biochemical investigations were carried out at Super Religare Laboratories (SRL) diagnostics across all study centers in India.

The investigational product i.e. Livrite marketed by Medley Pharmaceuticals Ltd, Mumbai and approved by regulatory authorities. Informed consent was obtained from the patients and the post marketing surveillance was done in accordance with the clinical principles laid down in declaration of Helsinki [20].

A total 15 patients with alcoholic (09 patients) or viral hepatitis (06 patients) were enrolled from 13 hepatology clinics in India. At the time of entry into the study, base-line demographics, clinical history, physical examination and biochemical evaluation were carried out. Patients were monitored for clinical signs and symptoms the end of 1st month of treatment, then subsequently 2nd and 3rd month of the treatment. Due to the ease and economic reasons of the patients, evaluation of liver function tests (LFT) was carried out at baseline and at the end of 3rd month of the treatment.

Inclusion Criteria: Both male and female patients over 15 years of age were included in this study.

Exclusion criteria: Patients with chronic active viral hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, liver cirrhosis, hepatocellular carcinoma were excluded from this trial. Patients were excluded if they had evidence of decompensated hepatic cirrhosis, a positive HIV antibody test result or positive result for HBsAg (surface antigen of the hepatitis B virus), or had used milk thistle products within the previous 30 days. Liver biopsy was not required for entry, although if obtained, the presence of moderate steatosis or steato-hepatitis were considered exclusions.

Intervention: Patients were treated with Livrite tablets (FDC of silymarin 210 mg, alpha lipoic acid 200 mg, N acetyl cysteine 200 mg and selenium 100 mcg) orally twice daily for three months.

Efficacy and Tolerability Evaluations: Primary efficacy variables includes change in LFT parameters such as, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), total bilirubin and bilirubin direct,total proteins & albumin. Safety outcomes included adverse events, which were recorded prospectively throughout the study. The patients were interviewed and asked for any type of adverse events throughout the study.

Statistical Analysis: The statistical analysis of LFT was carried out by using graph pad prism 5. Comparison between the baseline values and the values after 3rd month of treatment were made by paired T test. Values of $p < 0.05$ were considered statistically significant.

RESULTS:

A total of 15 patients made of 14 males and one female were enrolled and all of them completed the study. The age range of the patients was 15 to 50 years.

Aspartate aminotransferase (AST): Treatment with Livrite resulted in significant reduction in AST from baseline to the end of 3rd month and this difference was observed to be statistically significant (Figure 1). (369.9 ± 128.0 to 97.00 ± 34.27U/L; $p < 0.0001$);

Alanine aminotransferase (ALT): Treatment with Livrite resulted in significant reduction in ALT from baseline to the end of 3rd month and this difference was observed to be statistically significant (Figure 2). (652.93 ± 214.57 to 194.40 ± 82.51U/L; $p < 0.03$);

Alkaline Phosphatase (ALP): Treatment with Livrite resulted in significant reduction in ALP from baseline to the end of 3rd month and this difference was observed to be statistically significant (Figure 3). (197.47 ± 25.57 to 151.60 ± 17.92U/L; $p < 0.0059$);

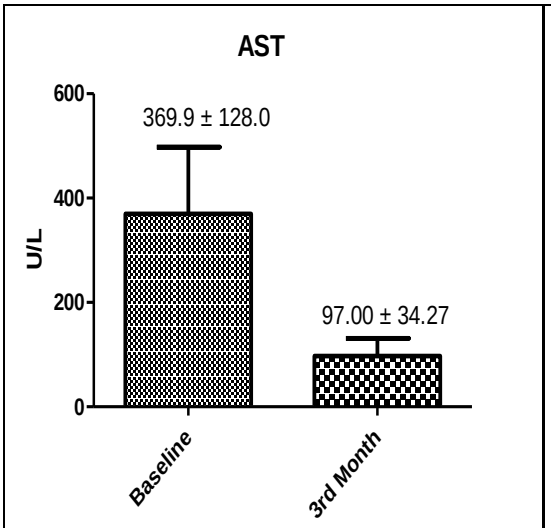


Figure 1: Change in AST from baseline

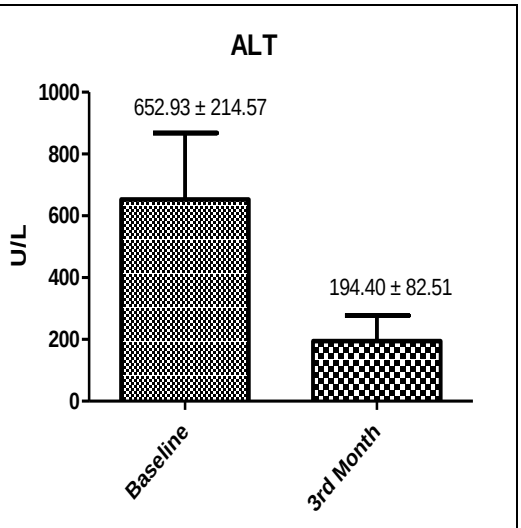


Figure 2: Change in ALT from baseline

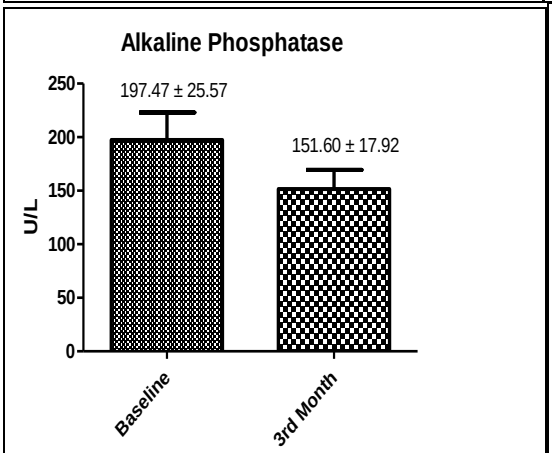


Figure 3: Change in Alkaline phosphatase from baseline

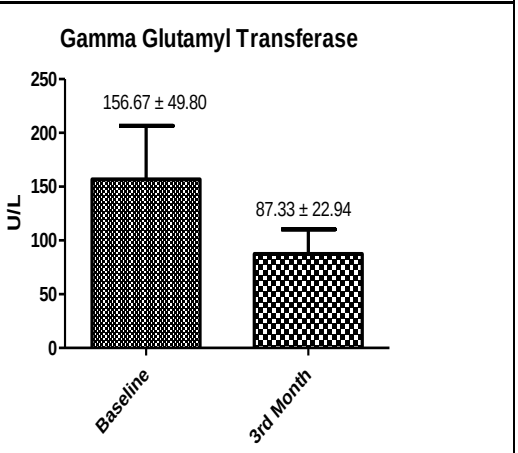


Figure 4: Change in G Glutamyl Transferase from baseline

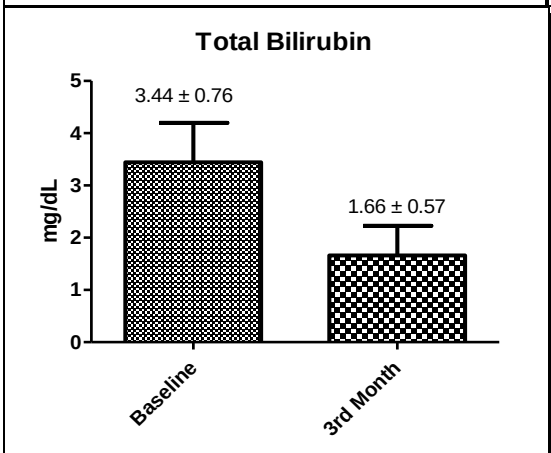


Figure 5: Change in total bilirubin from baseline

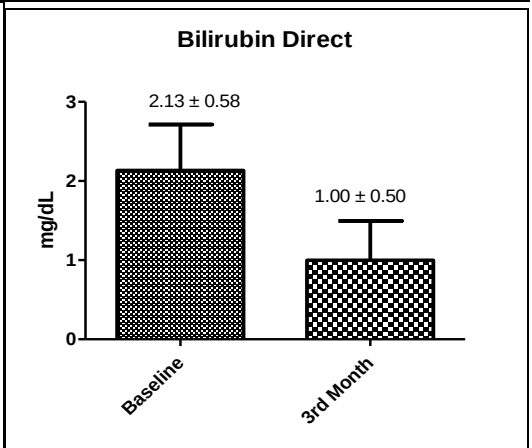


Figure 6: Change in Bilirubin direct from baseline

Gamma Glutamyl Transferase: Treatment with Livrite resulted in significant reduction in GGT from baseline to the end of 3rd month and this difference was observed to be statistically significant (Figure 4). (156.67 ± 49.80 to 87.33 ± 22.94U/L; $p < 0.0490$);

Total Bilirubin: Treatment with Livrite resulted in significant reduction in total bilirubin values from baseline to the end of 3 months; and this difference was observed to be statistically significant (Figure 5). (3.44 ± 0.76 to 1.66 ± 0.57 mg/dL; $p < 0.0192$)

Bilirubin Direct: Treatment with Livrite resulted in significant reduction in bilirubin direct values from baseline to the end of 3 months; and this difference was observed to be statistically significant (Figure 6). (2.13 ± 0.58 to 1.00 ± 0.50 mg/dL; $p < 0.0273$);

Total Protein & Albumin: The effect of Livrite on LFT parameters such as total protein and albumin was not significant. Total protein (baseline-7.29±0.54; after 3 months-7.32±0.67) g/dL Albumin: (at baseline 3.83±0.76 Vs. after 3 months: 4.11±1.03) g/dL.

Safety Evaluation: The patients were interviewed during each visit and at the end of the study for the presence of any adverse events. Adverse events were reported in 2 patients and clinicians recorded the severity of adverse events as of mild. The reported adverse events were bloating, dyspepsia, nausea and diarrhoea. None of the patient had history of allergy or hypersensitivity to any component of Livrite. However, as per clinicians decision, treatment with Livrite was neither withheld nor stopped, it was continued throughout the course of the trial, and adverse events were resolved completely.

Evaluation of Global efficacy and tolerability: As per clinicians assessment about efficacy and tolerability of FDC of silymarin, alpha lipoic acid, N-acetyl cysteine and selenium (Livrite), all patients tolerated the treatment well (except for 2 patients out of 15 who reported mild GI related adverse events) and were also benefitted in terms of improvement in LFT Parameters.

DISCUSSION:

Oxidative stress and associated damage represents the most common link between various forms of chronic liver injury and hepatic fibrosis. For example, oxidative stress leading to lipid peroxidation resulted due to oxidative stress is one of the crucial factors involved in the progression of nonalcoholic steatohepatitis (NASH) and liver cancer [21]. Lipid peroxidation is further triggered by viral infections or chronic alcohol abuse [21]. Alcohol abuse enhances the generation of ROS, decreases cellular antioxidant levels, and elevates oxidative stress in many tissues, prominently the liver. Primary mechanism by which ethanol produces hepatocyte injury is the oxidative stress induced by ethanol [22]. Since oxidative stress is a common pathogenetic mechanism contributing to progression of hepatic damage in inflammatory liver disorders, including acute and chronic hepatitis, antioxidants are understandably

considered as a crucial therapeutic approach for the management of these liver disorders.

Several natural compounds have the potential to scavenge the ROS molecule, thus reducing oxidative stress directly, or they may offer an indirect protection by activating endogenous defense systems [23]. Therefore, optimum doses of primary antioxidants should be the initial therapeutic strategy to restore and thereafter to maintain the serum and tissue concentrations of normal antioxidant values.

Silymarin is one of the oldest and intensively researched medicinal plants in the management of liver diseases [24]. Silymarin is a free radical scavenger that interacts directly with the cell membrane components to prevent any abnormalities in the content of lipid fraction responsible for maintaining normal fluidity. One of the mechanisms that can explain the capacity of silymarin to stimulate liver tissue regeneration is the increase in protein synthesis in the injured liver [24].

Silymarin's hepatoprotective action is due to the ability to increase the cellular content of GSH, regulation of membrane permeability and to increase membrane stability; stimulation of ribosomal RNA polymerase and subsequent protein synthesis, leading to enhanced hepatocyte regeneration, enhanced glucoronidation and protection from glutathione depletion, thus producing immunomodulatory effects 24].

A double-blind controlled study by Salmi and Sarna evaluated the effect of silymarin in 106 patients with alcoholic liver disease [25]. All patients had elevated serum transaminase levels (ALT and AST) and 90 had confirmed histological diagnosis. The patients were randomly allocated to either silymarin or placebo group and the duration of the trial was 4 week. A highly significant decrease in ALT and AST levels was observed with silymarin when compared with placebo [25].

In a Hungarian study of 36 patients, with chronic alcoholic liver disease, a dose of 420 mg/day of silymarin resulted in normalization of serum transaminases (AST, ALT and GGT), total bilirubin and an improvement in the histological examination of liver biopsies after 6 months of treatment [26].

Pares et al studied the effect of silymarin in alcoholics with liver cirrhosis with respect to their survival, clinical and laboratory changes [27]. This randomized double blind multicenter study compared 450 mg/day silymarin in three divided doses (n=103) with placebo (n=97), enrolled 200 alcoholics with histologically or laparoscopically proven liver cirrhosis. The primary outcome was time to death and the secondary outcome was progression of liver failure. Survival was similar in patients receiving silymarin or placebo and was not influenced by the gender, the persistence of alcohol intake, the severity of liver dysfunction or by the presence of alcoholic hepatitis in the liver biopsy [27].

Another component of the FDC used in our study is NAC which is frequently utilized where intracellular oxidant-antioxidant balance is concerned. A recent study has reported a significant decrease in liver steatosis and fibrosis

in patients with NASH receiving Metformin and NAC [28]. M Khoshbaten et al [28] performed another study in 30 patients with non-alcoholic fatty liver disease (NAFLD) and randomly assigned to receive either N-acetylcysteine or vitamin C. It was observed that a three-month supplement of NAC improved ALT and the size of the spleen in patients with NAFLD [28].

Selenium is essential trace element and a component of glutathione peroxidase and other peroxidases. Selenium is integrated into selenoproteins that have a broad range of pleiotropic effects, ranging from antioxidant to anti-inflammatory effects. Higher selenium status or selenium supplementation has also shown to have antiviral effects [29]. Moreover, selenium is an active immunomodulator, much more potent anti-oxidant than vitamins A, C, E and beta-carotene [17, 18].

Unlike other types of antioxidants, alpha lipoic acid is soluble in both fat and water, which allows the protection of cells throughout the body (both lipid and water components). It recycles other constitutional antioxidants (vitamin C, vitamin E, and glutathione). Furthermore, important coenzyme for the production of acetyl coenzyme A (important in liver cell metabolism) is alpha lipoic acid. Alpha lipoic acid reduces the levels of ethanol induced metabolic breakdown products, and thus may be an effective treatment for alcohol induced hepatitis & early cirrhosis [11].

Berkson BM et al [30] reported that the triple antioxidant combination of alpha lipoic acid, silymarin and selenium for a conservative treatment of hepatitis C because these substances protect the liver from free radical damage, increase the levels of other fundamental antioxidants, and interfere with viral proliferation. The 3 patients presented in their research followed the triple antioxidant program and recovered quickly and their laboratory values remarkably improved. Furthermore, liver transplantation was avoided and the patients were back at work, carrying out their normal activities, and feeling healthy [30].

Hepatotoxicity is a serious adverse drug reaction in tuberculosis (TB) patients receiving anti-TB drugs (isoniazid, pyrazinamide and rifampicin) and is one of the most challenging clinical problems worldwide [31]. Studies have shown that anti-TB-drug induced hepatotoxicity is primarily due to oxidative stress, caused by the anti-TB drugs and its metabolites. According to Funde et al. [32] oxidative stress due to free radical generation and subsequent lipid peroxidation of membrane play a crucial role in the pathogenesis of drug induced liver injury. Results obtained in the present study are comparable to the previous studies [25-28, 30]. With the use of silymarin, N-acetyl cysteine, alpha lipoic acid and selenium, this study showed significant improvement in the LFT parameters namely, AST, ALT, ALP, GGT, total and direct bilirubin at the end of 3 months. Based on the result of this trial, it can be concluded that, Livrite (FDC of silymarin, alpha lipoic acid, n-acetyl cysteine and selenium) demonstrates a hepatoprotective effect and thus has therapeutic usefulness in alcoholic and non-alcoholic disorders that involves oxidative stress.

Furthermore, hepatoprotective properties of Livrite can also be utilized to reduce the hepatotoxic effects attributable to anti-TB chemotherapy and to enhance the patient adherence to the treatment.

Clinicians would like to point out the three limitations of this present study: (1) the study was designed in an open-label fashion with no comparator arm; (2) the three month study duration may not have been long enough to assess long-term results; and (3) the sample size was relatively small. However, the positive results of the present study justify the need of larger double blind studies.

CONCLUSION:

From this study it could be postulated that antioxidants would be therapeutically effective under circumstances of elevated oxidative stress as in case of alcoholic liver disease, hepatitis & NASH or in cases exposed to a stressor that generates exacerbated oxidative injury like anti tuberculosis drugs. The FDC of silymarin, alpha lipoic acid, N-acetyl cysteine and selenium (Livrite) significantly decreased the ALT, AST, ALP, GGT, total and direct bilirubin level after 3 months of treatment from the baseline which shows the plausible benefits of anti-oxidant therapy in the management of hepatic disorders.

ACKNOWLEDGEMENTS:

Authors acknowledge the support of all the following doctors for providing their observations. These are, Dr. K.K. Tiwari, Dr. Rajiv Hambarde, Dr. Dilip Gogoi, Dr. Ch Arunkumar, Dr. S.K. Gupta, Dr. Samir Gupta, Dr. A.T.M. Saddiqui, Dr. Avik Roy Choudhury and Dr. M. Sanwarwalla. Authors are thankful for the immense help received from Dr. Mahesh Baviskar M.D (Pathology) for evaluating and interpreting the pathology investigational reports. The authors are also grateful to Mr. Shailesh K. Pallewar (M.Pharm-Pharmacology) for providing the statistical analysis.

Conflict of Interest: All authors had access to the data and vouch for the veracity and completeness of the data and the data analysis. The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES:

1. Wang FS, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China Hepatology. 2014 Dec; 60 (6):2099-108
2. http://www.liver.ca/liver-disease/ Cited on November 2015
3. Premashis Kar. World Hepatitis Day-new challenges; Indian J Med Res. 2011 Jul; 134(1): 1–3.
4. Viral hepatitis eradication in India by 2080 - gaps, challenges & targets. Indian J Med Res 140, July 2014, pp 1-4
5. Salvador Manzo-Avalos and Alfredo Saavedra-Molina. Cellular and Mitochondrial Effects of Alcohol Consumption Int J Environ Res Public Health. 2010 Dec; 7(12): 4281–4304.
6. Morgan TR, Mandayam S, Jamal MM. Alcohol and hepatocellular carcinoma. Gastroenterology. 2004 Nov; 127(5 Suppl 1):S87-96.
7. Ali A Mokdad, Alan D Lopez, Saied Shahraz, Rafael Lozano, Ali H

Mokdad, Jeff Stanaway, Christopher JL Murray and Mohsen Naghavi. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis BMC Medicine 2014, 12:145

8. Sabyasachi Ray; Alcoholic Liver Disease (ALD), Chapter 71, Association of Physician of India, Medicine update 2007
9. B. Poljsak and I. Milisav. The Neglected Significance of "Antioxidative Stress". Oxid Med Cell Longev. 2012; 2012: 480895.
10. Halina Cichoż-Lach and Agata Michalak. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol. 2014 Jul 7; 20(25): 8082–8091
11. Ms. Mamta Singh, Dr. Seema Gupta, Dr. Rajesh Pandey, Dr. H.K. Aggarwal, Dr. S. K. Aggarwal. Oxidative Stress and Chronic Alcohol Liver Disease: the Current Perspectives. IAJPR.2014:4 (03) Page 1428-1446
12. Lu Sc. Antioxidants in the Treatment of Chronic Liver Diseases: Why Is the Efficacy Evidence So Weak in Humans? Hepatology. 2008 Nov;48(5):1359-61
13. Luminita Pilat, Ciprian Mihali, Hildegard Herman, Cristina Popescu, Violeta Turcus, Aurel Ardelean, Ardelean Gavril, Teodora Mariasiu, Calin Popa, Anca Hermenean. Pharmacology of Silybum Marianum and its Active Constituents. Therapeutic Activity – Part 1; Jurnal Medical Aradean (Arad Medical Journal) Vol. XIV, Issue 2, 2011, Pp. 25-33
14. Chad Kerksick and Darryn Willoughby. The Antioxidant Role of Glutathione and N-Acetyl-Cysteine Supplements and Exercise-Induced Oxidative Stress; J Int Soc Sports Nutr. 2005; 2(2): 38–44
15. Dworkin B, Rosenthal WS, Jankowski RH, Gordon GG, Haldea D. Low blood selenium levels in alcoholics with and without advanced liver disease. Correlations with clinical and nutritional status Dig Dis Sci. 1985 Sep;30 (9):838-44
16. Thuluvath PJ, Triger DR. Selenium in chronic liver disease. J Hepatol. 1992 Mar;14(2-3):176-82
17. Baraboĭ VA, Shestakova EN Selenium: the biological role and antioxidant activity. Ukr Biokhim Zh 2004 Jan-Feb; 76 (1):23-32.
18. Ozardali I, Bitiren M, Karakilçik AZ, Zerin M, Aksoy N, Musa D. Effects of selenium on histopathological and enzymatic changes in experimental liver injury of rats. Exp Toxicol Pathol. 2004 Oct; 56 (1-2): 59-64
19. Halina Cichoż-Lach, Agata Michalak. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol 2014 July 7; 20(25): 8082-8091
20. World Medical Association. Declaration Of Helsinki Ethical Principles for Medical Research Involving Human Subjects: www.wma.net/en/30publications/10policies/b3/17c.pdf assessed on November 2015
21. Hye-Lin Ha, Hye-Jun Shin, Mark A Feitelson and Dae-Yeul Yu. Oxidative stress and antioxidants in hepatic pathogenesis. World J Gastroenterol. 2010 Dec 28; 16(48): 6035–6043
22. Wu D, Cederbaum AI. Oxidative stress and alcoholic liver disease. Semin Liver Dis. 2009 May;29(2):141-54
23. Esrefoglu M. Oxidative Stress and Benefits of Antioxidant Agents in Acute and Chronic Hepatitis. Hepat Mon. 2012; 12(3):160-7.
24. Ghosh A, Ghosh T and Jain S. Silymarin-A Review on the Pharmacodynamics and Bioavailability Enhancement Approaches. Journal of Pharmaceutical Science and Technology Vol. 2 (10), 2010,348-355
25. Salmi HA, Sarna S. Effects of silymarin on chemical, functional and

morphological alterations of the liver. A double-blind controlled study. Scand J Gastroenterol 1982; 17: 517- 21.

26. Feher I, Deak G, Muzes G. Liver protective action of silymarin therapy in chronic alcoholic liver diseases. Orv Hetil 1989; 130 : 2723-7.
27. Parés A, Planas R, Torres M, Caballería J, Viver JM, Acero D, Panés J, Rigau J, Santos J, Rodés J. Effects of silymarin in alcoholic patients with cirrhosis of liver: results of a controlled doubleblind randomized and multicenter trial. J Hepatol 1998; 28 : 615-21.
28. Manouchehr Khoshbaten, Akbar Aliasgarzadeh, Koorosh Masnadi, Mohammad K Tarzamani, Sara Farhang, Hosain Babaei, Javad Kiani, Maryam Zaare, Farzad Najafipoor. N-Acetylcysteine Improves Liver Function in Patients with Non-Alcoholic Fatty Liver Disease. Hepatitis Monthly 2010; 10(1): 12-16
29. Rayman MP. Selenium and human health. Lancet. 2012 Mar 31;379(9822):1256-68
30. Berkson BM. A conservative triple antioxidant approach to the treatment of hepatitis C. Combination of alpha lipoic acid (thioctic acid), silymarin, and selenium: three case histories. Med Klin (Munich). 1999 Oct 15;94 Suppl 3:84-9
31. Khushboo Ambreen, Rolee Sharma, Kaleshwar Singh and Sudhir Kumar: Anti-Tuberculosis Drug-Induced Hepatotoxicity: A Review; International Journal of Advanced Biotechnology and Research(IJBR) Vol5, Issue3, 2014, pp423-437
32. Satyajeet K. Funde, Jugalkishore B. Jaju, Shrikant C. Dharmadhikari, Ganesh R. Pawar. Effect of Lagenaria siceraria fruit extract (Bottle gourd) on hepatotoxicity induced by antitubercular drugs in albino rats. Int J Basic Clin Pharmacol. 2013; 2(6): 728-734

LETTER TO THE EDITOR

ALLERGIC REACTIONS TO IMPRESSION MATERIALS

***[1]Shishir Ram Shetty, [1]Sura Ali Ahmed Foud Al- Bayati, [2]Shakeel Santerbennur Khazi, [3]Sesha Reddy, [4]Walid Shaaban El-Sayed and [5]Arun Sekharan Devarajan**

***[1]Department of Oral Medicine and Radiology, [2]Department of Prosthodontics, [3]Department of Periodontics [4]Department of Oral Biology, College of Dentistry, Gulf Medical University, Ajman, United Arab Emirates; [5]Conservative Dentistry, GMC Medical & Dental Specialty Centre - Sharjah**

***Corresponding author**: shishirshettyomr@gmail.com
Submitted: October 2015, Accepted: December 2015

Dear Editor

Materials used for dentistry for impression making procedures are generally safe. However on rare occasions anaphylactic reactions ranging from mild rashes to life threatening situations have been reported [1]. We have highlighted few rare reports of allergic reaction to impression materials. Alginate is one of the most commonly used impression materials. It is chemically extracted from seaweed. It is composed of salts of alginic acid (basically a complex polymer polysaccharide) [2]. In a survey conducted few decades back in British factories producing alginate, it was observed that 7.0% of the factory workers suffered from seaweed allergy [2]. According to the report 4.5% of the workforce had allergy to sodium alginate. When a challenge test for alginate was carried out these workers elucidated immediate airway obstruction [2]. In a recent case report a fatal reaction to alginate has been highlighted [2]. The patient had both cardiovascular and lung diseases hence epinephrine could not be used to treat the anaphylactic reaction. The postmortem toxicology report confirmed that the cause of death was anaphylactic shock secondary to aliginate used during dental impression procedure [1]. Contact with polymeric impression materials for long period may lead to allergic contact dermatitis. Cutaneous allergic test carried out on few dentist and dental assistants working with these materials for long time revealed strong evidence of allergy [3]. Animal experiments have shown that these polymeric impression materials have high capacity for sensitization and also potent irritants [3]. Two cases of allergic reaction to silicon based impression material have also been reported [4]. The sensitization to silicon was caused either due to previous impression procedure or environmental exposure. Both the

cases were confirmed by epicutaneous allergic testing [4].

Polyether impression materials have been used in dentistry for over five decades. Although reports of allergic reaction to polyether surfaced in the 1970s it decreased after a change of a catalyst used by manufacturer. However, reports of allergic reaction to polyether impression material reemerged again recently. On this occasion the cause was traced to a component of the base paste [5].

These were a few very rare instances when allergic reactions have been observed for impression materials. However, since there is a constant improvement and innovation in impression materials coupled with the use of newer chemical agents the chances of allergic reactions to impression materials although rare can never be ruled out completely.

REFERENCES:

1. Gangemi S, Spagnolo EV, Cardia G, Minciullo PL.Fatal anaphylactic shock due to a dental impression material.Int J Prosthodont. 2009 Jan-Feb;22(1):33-4
2. Henderson AK, Ranger AF, Lloyd J, McSharry C, Mills RJ, Moran F Pulmonary hypersensitivity in the alginate industry. Scott Med J. 1984 Apr; 29(2):90-5
3. Kulenkamp D, Hausen BM, Schulz KH. Contact allergy caused by new dental impression materials]. Hautarzt. 1977 Jul;28(7):353-8
4. Oliveti E and Hegedus C. Contact allergy reactions to Silodent impression material Fogory SZ 1994 Apr;87(4):115-9
5. Pauline M, Szeimies RM, Landthaler M, Schmalz G.A rare allergy to a polyether dental impression materialClin Oral Inv 2012; 16(4):1111-1116

INSTRUCTIONS FOR AUTHORS:

AIMS AND SCOPE:

Pacific Journal of Medical Sciences is a peer-reviewed, multidisciplinary journal published by the School of Medicine and Health Sciences (SMHS), University of Papua New Guinea (UPNG).

The aim of The Pacific Journal of Medical Sciences is to provide the forum for researchers, medical practitioners and other professionals to record, publish, and share ideas and research findings that serve to enhance the understanding of the aetiology, symptoms, diagnosis, prevention, control and management of human disease conditions world wide.

The Pacific Journal of Medical Sciences publishes original scientific research reports, case reports, short communications, letters to the editor and reviews, representing new and significant findings in all areas of medical, biomedical and health sciences (including epidemiology, public and environmental health). Book reviews, scientific news and conference proceedings are published on special request.

EDITORIAL POLICIES:

The Pacific Journal of Medical Sciences (Pac. J. Med. Sci.) editorial policies require that: All manuscripts accepted for publication must reflect novelty and originality of quality research and be of interest to a multidisciplinary audience. All papers submitted for publication are peer-reviewed by two anonymous reviewers and the editor-in-chief or a designated member of the editorial board.

The editorial board may request for review articles, commentaries or short reviews on contemporary medical or biomedical issues that the board considered important to the advancement of the aims of the journal.

Original research papers should be both complete and concise; they should essentially offer conclusive results, but they should not exceed 7,500 words, including abstract, tables, figures and references.

Short communications and reports should not exceed 3500 words, including abstract, tables, figures and references. Review articles should not exceed 6,000 words, including tables, figures and references. Letter to the Editor should be brief and to the point.

On preliminary editing, all manuscripts that fail to meet the basic requirements indicated above and those that contain significant and obvious typographical errors are returned without further processing.

Manuscripts submitted will be reviewed and considered for publication only if they have not been published, simultaneously submitted or

already accepted for publication in another journal. The author responsible for correspondence must show evidence of approval of all co-authors when submitting a paper for publication.

All relevant ethical approval for research involving human and animal subjects must conform to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

Only research methods that comply with internationally accepted principles of humane animal experimentation are accepted for publication in The Pacific Journal of Medical Sciences. The decision to accept, revise or reject any manuscript for publication in The Pacific Journal of Medical Sciences is the responsibility of the editor-in-chief; this is done after reviewing the reports and comments from the reviewers, in consultation with members of the editorial board.

Disclaimer:

All statements and opinions expressed in any of the manuscripts published in The Pacific Journal of Medical Sciences are of the authors and co-authors, and not necessarily of the editors or members of the editorial board. The editor-in-chief and members of the editorial board of The Pacific Journal of Medical Sciences disclaim any responsibility or liability for such material and do not guarantee or endorse any products or services mentioned in the articles, nor guarantee any claims made by the authors of the articles.

SUBMISSION OF MANUSCRIPT:

Manuscript should be written in clear and concise English and be intelligible to those that are not specialists in the particular scientific area. Manuscript that does not satisfy these requirements but is acceptable for publication in the Pacific Journal of Medical Sciences because of its essential scientific content will be returned to the authors for extensive and appropriate revision, as recommended by the reviewers and editors.

A covering letter to clarify the following should accompany any manuscript submitted for publication in the Pacific Journal of Medical Sciences: (a) That the scientific data contained in the manuscript has not been published or submitted for publication in any other journal; (b) That ethical clearance and permission for the research had been obtained from the appropriate committee(s) in the institution(s) where the work was carried out; (c) That all the authors have read and approved the content of the manuscript; (d) The name, address and email contact of the author responsible for correspondence and for communicating with others about revisions and final approval of proof of manuscript; (e) Statements quantifying the contribution of each author to the manuscript (*this is a requirement in the latest*

guidelines of the International Committee of Medical Journal Editors);

Only electronic copy of the manuscript sent as e-mail attachment should be submitted using the approved format indicated in the appropriate sections of this document.

Manuscript should be sent by email to any of the following: pacjmedsci@gmail.com.; templevictor@gmail.com;

PREPARATION OF MANUSCRIPT:

Manuscripts should be prepared on one side of A4 paper, using double-spacing. Pages are to be numbered consecutively in the bottom right-hand corner. Manuscript should include the following sections: Title page, abstract and keywords, text, acknowledgements, references, tables and figures.

Style: The Pacific Journal of Medical Sciences uses both UK and US spelling. Only one or the other should be used throughout a manuscript. SI units should be used for all measurements. Use abbreviations to avoid repetition of long technical terms: Indicate the abbreviation in parentheses when the word is used in full for the first time. Use the approved generic names of chemical substances and drugs. Do not use trade names or brand names of chemicals and drugs.

Title page: The following should be on the title page: (a) Title of the manuscript – it should be concise and informative; (b) Short running title of not more than 40 characters (optional); (c) Name of each author (first name, middle initial and last name), including highest academic degree; (d) Name and address of institution(s) in which the work was carried out; (e) Name, postal address and email contact of the author responsible for correspondence; Source(s) of research or other types of support for the research project, if any,

Abstract and key words:

The abstract should not be more than 300 words. The following should be clearly stated in the abstract: the purpose of the study, basic procedures, main findings (specific results and statistical significance, if any), and principal conclusions. Abbreviations and references should not be included in the abstract. Not more than 8 key words should be put below the abstract. Key words are used to assist indexers in cross-indexing published articles and may be published with the abstract. Medical Subject Headings (MeSH) list of the Index Medicus should be used for selecting key words (www.nlm.nih.gov/mesh/meshhome.html)

Text:

Text of an original manuscript should be separated into the standard IMRAD format as follows: Introduction, Materials and Methods, Results, Discussion. Sections on Acknowledgements and References should be included.

Introduction: This section should: (a) summarize relevant previous work, using appropriate references, without any extensive review of the subject; (b) clearly state the purpose of the study and summarize the rational for the study or observation; (c) avoid given any data on the work being reported.

Materials and Methods: This section should: (a) clearly indicate either the sampling procedure or observational subjects; (b) give appropriate references for established techniques and procedures; (c) new techniques and procedures and extensive modifications of existing ones should be presented in sufficient details so that other researchers can easily reproduce and evaluate them; (d) indicate appropriate quality control procedures used for laboratory methods and techniques; (e) indicate ethical procedures if either human subjects were involved [if informed consent was obtained from each subject] or if appropriate guide line for using laboratory animals were followed [see editorial policies above]; (f) indicate statistical methods used, if any.

Results: Data obtained should be presented in logical sequence in the text, tables and figures should be adequately explained to facilitate their interpretation. Avoid duplicating the results by repeating in the text all the data presented in the tables and figures. The text in the results section should only emphasize or summarize the important data.

Discussion: Major findings should be highlighted before the minor findings. All findings should be related to other relevant studies, if any, using appropriate references. Indicate the implications of the findings and their significance or limitations, including implications for future research. When warranted, propose new hypotheses with appropriate data, but be sure to clearly label them as such. The conclusions should be linked with the goals of the study and be clearly supported by evidence / data. Include recommendations, if applicable.

Acknowledgements:

The following should be acknowledged: Research or other financial grants; Material support, Contributions of Institutions, Colleagues, and other relevant participants.

References:

The Pacific Journal of Medical Sciences uses the Vancouver system of referencing. The references should be numbered, using Arabic numerals in square brackets, in the order in which they are first used in the text, tables, figures, and legends. In the reference section, list the references in the order of appearance in the text, tables, figures and legends. ***Abstracts, unpublished data, oral communications, and personal communications should not be included in the reference section***. All references should

be verified against the original documents. In the reference section, the names of all authors should be included. Avoid using "et al." in the reference section. Names of journals should be abbreviated, using the approved style indicated in Index Medicus/PubMed. References should be listed according to the examples given below:

Journal articles:

Brander LC, Buess H, Haldimann F, Harder M, Hanggi W, Herrmann U, Lauber K, Niederer U, Zurcher T, Burgi U, Gerber H. Urinary iodine concentration during pregnancy in an area of unstable dietary iodine intake in Switzerland. J Endocrinology Invest. 2003, 26 5: 389 – 396.

Book:

Gillett JE. The health of women in Papua New Guinea. PNGIMR: Kristen Press, 1991

Chapter in a Book:

Chaney SG. Principles of nutrition II: Micronutrients. In: Delvin TM, editor. Textbook of Biochemistry with Clinical Correlations, 4th ed. Brisbane: Wiley-Less, 1997: 1107– 36.

Published proceedings paper:

Kruse-Jarres JD. Basic principles of zinc metabolism. *In*: Kruse-Jarres JD, Scholmerich J, editors. Zinc and diseases of the digestive tract. Proceedings of the International Falk workshop, Germany, 1996: 3 – 15.

Tables:

Tables should be numbered sequentially in Arabic numerals, typed double-space on separate A4 paper for each table; vertical lines should not be used to separate columns. Each table should be self-contained with a comprehensive but concise legend/heading; column headings should be brief, with units in parenthesis. All non-standard abbreviations used in tables should be explained in footnotes, using the following symbols in this sequence: *, §, ¶, #, $.

Illustrations:

Graphs, line drawings, bar charts, maps, etc., should be labelled as 'figures' and numbered consecutively, using Arabic numerals. All figures should be drawn using computer graphics. Legends should be brief but understandable without referring to the text. Photographs should be unmounted sharp, glossy black and white prints. Photographs should contain scale bars, not magnifications. Colour photographs are not acceptable. Figures, reproduced from another source, should be clearly indicated and appropriate references and written permission from the copyright holder must be submitted with the manuscript.

Electronic copy of manuscripts (e-mail):

When a manuscript is accepted for publication, the corresponding author will be required to send an electronic copy of the corrected or

modified manuscript by email. All email attachments should be scanned with anti-virus software before sending. Automatic software for referencing, footnotes, headers, footers, etc., should not be used during formatting.

All manuscripts should be formatted using MS WORD.

GALLEY PROOFS:

Galley proof will be sent by email to the correspondent author. Only minor corrections should be made, no major alterations to the manuscript will be accepted. Galley proof should be returned within maximum 5 working days from the date of receipt. If any major modification is made, the manuscript will be rejected at this stage. Correspondent author should correct all printing errors and ensure that the typesetting is accurate in the galley proof. Note that the correspondent author, not the publisher will be responsible for any such errors, should they occur in the published paper.

REPRINTS:

Reprints will not be sent to authors, because the PJMS is an on-line journal. The Web-link will be sent by email to the corresponding author. One copy of a paper may be sent to the correspondent author on request only, subject to availability of funds to cover postage. Authors may download and print the journal at their own expense.

COPYRIGHT:

Manuscripts accepted for publication in The Pacific Journal of Medical Sciences become the property of the journal. Therefore, all authors **may** be requested to sign a transfer of copyright form and send scan copy by email attachment to the editorial office. The copyright gives the journal the exclusive rights to reproduce, translate and distribute the article for academic purposes.

CHECKLIST FOR AUTHORS:

- Running title included (Can be omitted if not applicable);
- Abstract prepared according to instructions and include key words;
- Manuscript typed double-space on one side of A4 paper;
- References cited in square brackets in text and listed according to approved style for this journal; Uniform spelling throughout the text;
- Tables and Figs on separate A4 pages;
- Covering letter written as required by this journal;
- E-mail address of corresponding author indicated.