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Research Ethics in Developing Countries: Issues, Opportunities and Challenges

Waris Qidwai¹, Shahan Waheed² and Ammad Fahim³

Research is necessary for progress and mandatory for the success of any nation. With the advancement in science and technology in today's world, maintaining highest ethical standards in research is becoming a challenging task and increases responsibilities of investigators.¹ Protection of human subject's rights is of paramount importance in conducting any research involving human beings.²

Research on human subjects is becoming increasingly challenging in developed world as research subjects are more aware of their rights and less willing to participate unless well compensated. This has made research on human subjects in developed world very expensive and investigators/industry and research sponsors are increasingly looking towards the developing world to conduct research as it is less expensive. This has resulted in very serious concerns being raised by various stakeholders to ensure the rights of human subjects are protected.³

Research subjects in the developing world are a vulnerable population and require additional safeguards for protection of their rights. Research subjects in the developing countries are not very much educated and therefore it is important that written Informed Consent form administration is properly conducted. It should be in simple and preferably local language.⁴ The argument that less educated research subjects cannot be made to understand research details is not true. Every effort should be made that the research subject understands the nature of the research project, the risks involved and benefits offered if any. The right to participate or not to participate should be clearly written and made clear to the research subject. The approval of written Informed Consent form by Institutional Review Board (IRB) or Ethical Research Committee (ERC) should be mandatory to ensure it guarantees research subjects rights. Signature of a witness not from the research team should be taken to ensure research subject protection.

Financial incentives to relatively poor research subjects from the developing countries can compromise their rights and safety.³ It should be the responsibility of ERC to ensure

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that those with limited financial standing are not exploited and lured into taking risks without their clear understanding and appropriate compensation/insurance. Conducting human subject research in developing countries can be an incentive for Investigators as clinical drug trials can be done at a lower costs compared to those conducted in the developed countries.

At one end there are issues of safety of research subjects due to their vulnerability and on the other hand there are opportunities of bringing in research into the developing countries that has its own advantages. Research happening in the developing countries leads to capacity building⁵ in research of local human resource and they also get the opportunity to get on to the map of research activities going on around the globe. It brings in training opportunity for human resource in research in these countries and also enhances their earning capacity at an individual/institutional level. Researchers from these countries can learn from experience and training opportunities available in the developed countries as a result of research collaboration.⁶ Developing countries are therefore able to enhance their revenue generation as a result of research activities.

There are issues with regards to infrastructure and human resource availability for the conduct of human subject research of high quality in developing countries. Infrastructure is lacking altogether or is of poor quality hindering quality human subject research. Funds are required to invest in infrastructure development and maintenance in developing countries. Human resource with proper training, certification and experience to conduct high quality human subject research is lacking. Brain drain with migration of suitably qualified researchers to develop countries prevents whatever human resource exists in developing countries. Consistent government policies in support of promoting and encouraging human subject research are required.

Presence of academia is required to provide the necessary expertise necessary for human subject research in developing countries. One of the most important prerequisite for ensuring human subject research rights and safety is the establishment and presence of IRBs or ERCs at level of various academic or research institutions. Such IRBs should comprise of individuals who are suitably qualified and experienced to address ethical issues and safety of human research subjects. Such boards should have individuals from varied backgrounds including law, social sciences and religion in addition to medical and nursing. Members of IRBs should be of proven credibility and should not participate in the discussions concerning research protocols where they may have conflict of interest.

Standard of care given to a research subject in human subject research carried out in developing countries has been a matter of grave concern. Researchers should provide the best available care to the research subjects according to some while others argue that it should be the best available care in the research settings. This subject has been open to discussion and debate.

Collaboration between institutions/individuals from developed and developing countries is the way forward but should be done in a manner that is ethical and drives on the strengths of all stakeholders and results in mutually beneficial⁷ outcome for all involved. Developed countries offer the necessary human resource and necessary finance whereas developing countries offer availability of large human subject research opportunities. If interests of all concerned are taken into account in an ethical and mutually beneficial manner in the sharing and distribution of these resources and outcomes of research than it will result in a productive and long lasting relationship between these stakeholders.

In conclusion, research in developing countries should be collaborative, ethically sound and mutually supportive. It should protect human subjects and they should be adequately compensated for their participation.

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Effect of Fructose in Acetaminophen Induced Liver Injury in Rats

Fareeha Farooq¹, Abdul Khaliq Naveed², Naseem Saud Ahmad³, Adeel Arif⁴, Ijaz Ahmad² and Palwasha Waheed²

ABSTRACT

Objective: Low dose fructose was used in hepatotoxic rats to assess its hepatoprotective role. The objective of this study was to assess the effect of fructose on liver function using enzyme assays and morphologic changes.

Study Design: Quasi-Experimental study

Place and Duration of Study: Departments of Biochemistry, Pharmacology and Pathology, Army Medical College and National Institute of Health from Jan 2007–Jan 2008.

Methodology: One hundred and twenty healthy male Sprague-Dawley rats were injected Acetaminophen (APAP) (650 mg/kg) to induce acute hepatotoxicity, fructose (1g/kg) and *N*-acetyl cysteine (NAC) (1200 mg/kg) intraperitoneally. Blood samples were taken after ten hours and serum was separated and centrifuged. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, albumin and total bilirubin were measured using kit method. Liver biopsy was taken to observe the necrotic changes.

Results: APAP had 200% elevation of serum ALT and AST ($p < 0.01$). Serum alkaline phosphatase, bilirubin and albumin were insignificant as compared to controls in all the groups ($p > 0.05$). Fructose and APAP co-administration (group III) had insignificant effect on serum ALT ($p = 0.6$) and AST ($p = 0.9$) as compared to APAP group ($p > 0.05$). NAC (group IV) significantly decreased serum transaminases compared to groups II and III ($p < 0.01$). Fructose did not reduce centrilobular necrosis produced by APAP, while NAC had significant cytoprotection in this animal model.

Conclusion: Low dose fructose (1g/kg) has no hepatoprotective role in acute APAP hepatotoxicity *in vivo* and NAC conferred hepatoprotection. Additional studies are needed to understand the combined interaction of fructose and APAP, as fructose is being extensively consumed by general population in form of commercial beverages.

Key words: Acetaminophen, hepatotoxicity, fructose, rats.

INTRODUCTION

Acetaminophen is a cost effective antipyretic and analgesic prescribed to all age groups injudiciously. It is presumed to be safe against gastric erosions however an intake of 7g will cause poisoning that may be fatal if not treated timely.¹ Although it is a leading cause of hepatic failure in the western world, the data in Pakistan are lacking, where most of the population in rural areas has limited access to tertiary care hospital.

Therapeutic doses of APAP are metabolized by endogenous glucuronic acid, sulphate and reduced glutathione. A small amount of *N*-acetyl-*p*-benzoquinone imine (NAPQI) forms which is detoxified by glutathione.² Acute ingestion of 150-200 mg/kg (children) or 7 g total (adult) will cause excess production of NAPQI that will damage DNA, RNA and cellular proteins. It also damages mitochondria by creating pores in the inner mitochondrial membrane, called “mitochondrial permeability transition” (MPT). This initiates necrosis or apoptosis leading to cell death.³

Fructose in high doses cause obesity, hyperuricemia and metabolic syndrome.⁴ A low dose of fructose has been found protective to hepatocytes against APAP toxicity in cell culture studies.⁵⁻⁷ It decreased *N*-nitrosufenfluramine toxicity by providing more ATP by glycolytic pathway.⁸ A cardioprotective role of fructose was found by Jordan and co-workers in ischemia –reperfusion injury.⁹ Fructose improved neuronal function and studies are underway into neuroenergetics.¹⁰

In the light of biochemical observations and cytoprotective findings *in vitro*, the study was designed to evaluate hepatoprotective role of fructose in animal models of APAP toxicity.

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MATERIALS AND METHODS

Drugs and reagents

Fructose of analytical grade (Panreact: QUIMICA;Spain.), Acetaminophen (APAP) of pharmaceutical grade (Provas: Sami pharmaceuticals®, Pakistan) and *N*-Acetylcysteine (Parvolex; Mayne Pharma®, Wellington, New Zealand) were purchased. Commercial kits for ALT, AST, ALP, bilirubin (Linear Chemicals, Spain) and serum albumin (Diamate Technology, Spain) were purchased from Hamza dealers.

Animals

One hundred and twenty healthy male Sprague-Dawley rats weighing (180-220 g; 9-12 weeks of age) were obtained from National Institute of Health (Islamabad, Pakistan). The protocol was approved by the ethical committee of Army Medical College. Animals were acclimated for one week at the animal house of Army Medical College. Rats were kept at 23-25°C and 12 hr light/12 hr dark cycle. Animals had free access to water ad libitum and chow, before initiation of any treatment.

Rats were randomly divided into four groups ($n=30$ each). Control (group I), APAP (group II), APAP+ fructose (group III) and group IV received both APAP and NAC. Animals were kept on a 16 hrs fast before start of experiment with free access to water. APAP was given in a dose of 650 mg/kg intraperitoneally.¹¹ after 10 hrs. Group III received fructose (1 g/kg) in three divided doses at 0.5 hr, 4 hrs and 8 hrs (i .p.) after APAP injection. Group IV received *N*-acetylcysteine (1200 mg/kg, i. p) as a single dose half hour after APAP.¹² The control group 1 received vehicle alone.

Blood was obtained via cardiac puncture under ether anesthesia 10hrs after drug administration. It was allowed to stand for half hour at room temperature to get serum. Serum was then centrifuged at 1000 g for 10 minutes at 4°C and subjected to spectrophotometry using auto analyzer (Vitalab Selectra-E, Netherlands).

The portions of liver were stored in 10% formalin and then embedded in paraffin. Microtome sections of 5µm thickness were prepared from liver samples and subsequently stained with hematoxylin-eosin.¹³ These sections were examined for pathological findings. They were characterized as 0 with no sign; 1+ when only congestion was present; 2+ when vacuolar degeneration was seen and 3+ when predominantly centrilobular necrosis and inflammatory reaction was seen along with vacuolar degeneration.

STATISTICAL ANALYSIS

All the data were expressed as means \pm SEM. Comparison between groups was performed by one-way analysis of variance (ANOVA) followed by multiple comparison post-hoc tests using software SPSS V 11. Differences were considered significant at $p < 0.05$.

RESULTS

Serum ALT

The serum ALT value was increased from (mean \pm SEM) 46 \pm 4 IU/L in control group to 107 \pm 11 IU/L in group II ($p=0.001$). This value was 126 \pm 18 IU/L in group III which was higher but insignificant as compared to group II ($p=0.64$). *N*-Acetylcysteine decreased these levels towards normal with a value of 77 \pm 7 IU/ L in group IV as shown in figure1 ($p=0.27$).

Serum AST

In the control group, mean AST level was 175 \pm 11 IU/L. It increased to 364 \pm 24 IU/L by APAP treatment ($p=0.000$). Fructose and APAP co-treatment resulted in serum levels of 353 \pm 26 IU/L which was insignificant compared to group II ($p=0.97$). NAC treatment decreased the levels to 235 \pm 14 IU/L in group IV. It was significantly less when compared to both groups II and III ($p<0.0001$).

Serum Alkaline Phosphatase

The mean level of ALP in control rats was 161 \pm 4 IU/L. Treatment with APAP, fructose and NAC gave values of 150 \pm 5 IU/L, 147 \pm 4 IU/L, and 144 \pm 5 IU/L respectively. Comparison of group II with group I, III and IV was insignificant ($p=0.36, 0.97, 0.85$ respectively).

Serum Albumin

Mean serum albumin level was 41 \pm 0.4 g/L in the control group. The levels were 40 \pm 0.4 g/L in both the APAP and fructose treated groups. Treatment with NAC however decreased the value to 37 \pm 1.6 g/L. There was no significant difference in the mean values among the groups.

Serum Total Bilirubin

Serum bilirubin levels were 1.7 \pm 0.1 imol/L in the control group. APAP treatment produced insignificant change compared to control group with a value of 1.8 \pm 0.1 imol/L. Fructose and NAC gave values of 1.7 \pm 0.1 imol/L and 1.6 \pm 0.1 imol/L respectively. There was no significant difference in the mean values among the groups.

Histopathological Findings

In group II, APAP produced centrilobular necrosis, congestion of the central vein and sinusoids of the liver. An inflammatory infiltrate was also seen as indicated in figure 1. Fructose and APAP (group III) produced similar changes along with vacuolar degeneration. However these changes were localized to focal areas of centrilobular necrosis as shown in figure 2. In group IV, NAC and APAP co-administration produced congestion of the sinusoids and central vein along with vacuolar degeneration while centrilobular necrosis was seen in only four rats. This indicates that NAC was hepatoprotective in this setting.

DISCUSSION

In this study acetaminophen in group II caused significant hepatocellular damage as evident by increase in the serum transaminases of up to 200% during acute liver injury.¹⁴ The rise in serum ALT, AST, and ALP in Sprague Dawley rats was comparable with other studies. A higher value of ALT 607 ± 32 IU/L and AST 1178 ± 18 IU/L respectively has been reported with APAP given for twenty four hours against ten hours exposure in our study.¹⁵⁻¹⁷

There was no significant elevation of serum ALP, albumin and bilirubin in group II in our study, however a dose of 2 g/kg APAP produced a significant toxicity in another study. There was rise in ALP to 216 ± 9 IU/L and total bilirubin to $17 \mu\text{mol/L}$ after twenty four hours.¹⁸ Serum albumin in our control group was 41 ± 0.4 g/L which has already been reported in Sprague Dawley rats.¹⁹ Fructose has been shown to protect hepatocytes in hypoxia and anoxia in cell culture studies.⁵⁻⁷ The serum ALT of 126 ± 18 IU/L and AST of 353 ± 26 IU/L, serum ALP, albumin and bilirubin clearly indicates that fructose did not protect against APAP toxicity *in vivo*.

N-Acetylcysteine is an antioxidant which functions to regenerate glutathione. This decreases free radical damage and consequent cellular injury.²⁰ Serum ALT and AST levels in this group were 77 ± 7 IU/L and 235 ± 14 IU/L respectively (Table 1). It is significantly less as compared to both groups II and III ($p < 0.05$). Serum ALP, albumin and bilirubin were 144 ± 5 IU/L, 37 ± 1.6 g/L and $1.6 \pm 0.1 \mu\text{mol/L}$ respectively albeit not significant when compared to group II and III. Another study has reported ALT and AST of 41.1 ± 5.6 IU/L and 60.7 ± 9.6 IU/L respectively in albino wistar rats.²¹ They administered NAC intramuscularly for eleven days in a dose of 150 mg/kg while evaluating the drug cyclosporine A. When NAC was administered before inducing carbon tetrachloride toxicity in another study, it yielded certain beneficial effect. But similar to our results they were not able to reverse hepatic membrane damage completely.²²

The histopathological findings as indicated in table I confirm the APAP toxicity. The rise in serum enzymes by fructose and APAP is in accordance with the microscopic picture as shown in figure 1 and 2. It appears from the results of our study that fructose has different effects in cell culture studies and *in vivo*. Fructose did not decrease APAP toxicity while NAC protected from centrilobular necrosis in twenty six out of thirty rats.

The protection by fructose in cell culture studies was shown due to more ATP production via glycolysis. One of the proposed mechanisms is that it bypasses the rate limiting enzyme, phosphofructokinase I and stimulates pyruvate kinase. It also phosphorylates faster than glucose because K_m of fructokinase is less than glucokinase.^{7,8} In this study the dosing of fructose was done every four hours to maintain a constant supply of ATP, based on the work of Latta et al.

They showed that initial depletion of ATP by fructose 1-phosphate recovers by anaerobic glycolysis at 4 hrs.²³ Fructose would protect when the rate of production of ATP via glycolysis overcomes the rate ATP consumption during initial fructose 1-phosphate formation. It appears that insufficient ATP was generated to sustain ATP dependent membrane functions evident by raised transaminases in group III.

The results from this study are more relevant to the human conditions, in typically consumed concentrations and in typically consumed form in contrast to other animal studies. We find strong recommendations on this in recent literature.²⁴ These findings have implications, both specific to acetaminophen and can be interpreted in context of a general toxic liver injury. Although fructose did not protect from acute Acetaminophen liver injury, the fact that it did not increase liver toxicity may show that it can be safely taken by patients of liver injury. Recent evidence is growing in favour of a dose and duration dependent effects of fructose, and our study has similar conclusions.²⁵ This consideration is important in defining nutrition policy and consumer perceptions in the present era when the general public knows a lot on dietary sugars through media.

The understanding of pathophysiology of APAP is developing into newer concepts of apoptosis and MPT. Although a limitation of this study is lack of simultaneous measurement of ATP, apoptosis and MPT, however, this study does show that additional *in vivo* studies are needed on the role of different doses and duration of fructose in APAP toxicity.

CONCLUSION

This study demonstrated that a low dose fructose (1g/kg) has no hepatoprotective role in acute APAP hepatotoxicity when compared with NAC. Additional studies are needed to understand the combined interaction of fructose and APAP as both are being extensively consumed by general population.

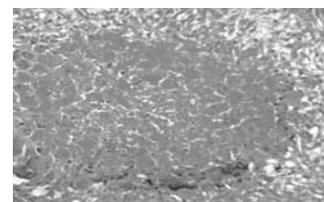


Figure 1: Photomicrograph (400 x magnifications) of liver showing necrotic area in group II rats treated with acetaminophen only.

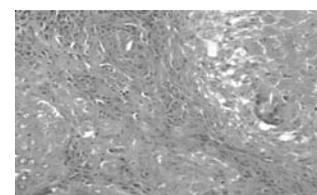


Figure 2: Photomicrograph (400 x magnifications) of liver showing necrotic area on right in group III receiving both acetaminophen and fructose showing similar centrilobular necrosis.

Table I: Liver function enzymes and histopathological findings with fructose and NAC in acute APAP toxicity

Group (n=30)	Serum liver enzymes (Mean ± SEM)					Histopathology			
	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Albumin (g/L)	Bilirubin (µmol/L)	0	1+	2+	3+
I Control	46 ± 4	175 ± 11	161 ± 41	41 ± 0.4	1.7 ± 0.1	28	2	-	-
II APAP	107 ± 11	364 ± 24	150 ± 5	40 ± 0.4	1.8 ± 0.1	-	-	-	30
III APAP+ fructose	126 ± 18**	353 ± 26**	147 ± 4	40 ± 0.4	1.7 ± 0.1	-	-	-	30
IV APAP+NAC	77 ± 7*	235 ± 14*	144 ± 5	37 ± 1.6	1.6 ± 0.1	-	-	26	4

** p>0.05 when group II is compared to group III which shows insignificant change.

* p< 0.05 when group IV is compared to groups II and III and indicates hepatoprotection.

0: No sign; 1+ congestion; 2+ vacuolar degeneration; 3+ vacuolar degeneration, centrilobular necrosis, and inflammatory reaction

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Spectrum of Unnatural Deaths in Hyderabad: An Autopsy Based Study

Ghulam Mustafa Yousfani and Muhammad Umar Memon

ABSTRACT

Objective: To determine the spectrum of unnatural deaths, focusing on gender vulnerability, manners of death, inhabitant, weaponry and seasonal prevalence for criminal episodes.

Study Design: Prospective study.

Duration and setting: Medicolegal Section of Liaquat University Hospital Hyderabad from January 2006 to December 2008.

Methods: Six hundred and ninety seven cases of unnatural deaths referred by police for autopsy were included. Those who died in hospital due to natural causes or complications of surgery were excluded. The variables considered were gender, manners of death, weaponry, inhabitant and seasonal variations in criminal deaths. Findings were expressed in numbers and percentages.

Results: Out of total 697 medicolegal autopsies conducted males were (595) 85.4% and females (102) 14.6%. Accidental deaths (471) 67.58% outnumbered homicidal (219) 31.42% and suicidal (7) 01% manners of death. Weaponry for assault preferred was firearms followed by hard blunt weapons. Peri-urban inhabitant victims of unnatural deaths were (314) 45% followed by those belonging to rural areas (209) 30% and urban dwellers (174) 25%. Unnatural deaths occurred more in summer than in winter season.

Conclusion: Majority of the victims of unnatural deaths were males dying due to accidental manner of deaths. Peri-urban victims out numbered the rural and urban dwellers. The fatalities were found in summer season more than in winter.

Key words: Unnatural deaths, Autopsy, Forensic Medicine.

INTRODUCTION

Nobody likes death inspite of the fact that death is an undeniable eventuality of life. Every living object has to taste the death.¹ Death investigation is a feature of modern day's civilized system of society. The causes and factors responsible for bringing about the fatal outcome require thorough investigation. To observe the failure of the medical treatment offered to the deceased or to rule out the factor of negligence of the caring team and to see the role of violence if any, autopsy remains the most appropriate medical tool of investigation. The former is a matter of interest to medical researchers, whereas later is matter of concern for the State.

Globally, the homicide accounts for 1.6 million deaths² and at least 1 million people are estimated to die from suicide world wide.³ Nearly 1.2 million road traffic accidents deaths occur globally each year as reported by WHO.⁴ There is no precise Registry based statistical data

representing Pakistan's scenario about prevalence of unnatural deaths, but studies done by various authors at different centers that represent data of a limited experience only, which has cumulative value.

If death is caused as a result of any terminal illness in the hospital or bedroom in peace and solace, everybody accepts it considering as a wish of God and mourns for the departed soul. On the contrary, if death, results as a consequence of violence, may it be self suffered by the deliberate act or negligence of others or as a corollary of an accident or misadventure, society displays resentment, cry for punishment to the responsible (s) and demand for future prevention from such incidents to happen. State apparatus, entrusted for receiving such information and investigations, instantly take cognizance of the happening and take appropriate actions accordingly. Such cases fall under the category of unnatural deaths.

Medico-legal autopsy acts as one of the investigative tool that helps the investigators in many ways.⁵ Positive identification of the deceased, determination of cause of death, time since death, manner of death, time between injury and death, and to discover and recover any clue(s) from the body within or without, that can possibly connect the crime with criminal or unearth the role of negligent act or omission in bringing about the fatality are some of the resultant beneficial outcomes of a medicolegal autopsy.⁶ Periodical publication of statistical data of unnatural deaths

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provide an insight about the trends of society and any change on either side in the attitude of people also gives an opportunity for social scientists and planners to formulate a strategy to combat crime to prevent it in order to extricate or minimize the loss of precious lives. This study is aimed to collect the data of unnatural deaths both criminal and non criminal in nature that occurred in the district of Hyderabad comprising of urban, peri-urban and rural areas and to compare it with the pattern of unnatural deaths occurring in other areas of Pakistan.

SUBJECT AND METHODS

This prospective study was conducted at the medicolegal section of Liaquat University Hyderabad for a period of three years, commencing from January 2006 to December 2008. This study included all the cases of death due to any form of violence and submitted for autopsy in the mortuary of Civil Hospital Hyderabad. Cases died of apparently natural causes in the Liaquat University Hospital and cases of infanticide, feticide and maternal deaths were excluded. As per rules medicolegal autopsies are conducted by authorized medical officers in the mortuary designated by the provincial government.^{7,8} The dead bodies were examined in a definite prescribed order viz examination of clothes, external body examination, dissection of the body to find out the cause, mode, time of death, the weapon/means responsible for bringing about the fatality and the survival time period between injury and death. If following this procedure, anatomical cause of death could not be ascertained, suspected tissues and body fluids were collected and submitted for detailed, histological and toxicological diagnosis.

The bodies were dissected by Robert Virshow's technique.⁹ All the body cavities were dissected, cranium, thoracic and abdominal cavities in that order and organs were examined grossly both in situ and after taking out of the body.

A Proforma was designed depicting deceased's gender, causative agent of death, manner of death and the area from where the bodies were brought and the calendar months indicating season, summer or winter.

RESULTS

Total unnatural deaths submitted for autopsy in three years period were 697, ranging from 212 to 250 cases in a year, whereas year 2007 saw the maximum number of cases that is 250. Male 595/697 (85.4%) remained the victim of unnatural deaths as compared to female 102/697 (14.6%) (Table: I). The ratio remained 6:1. Amongst the deaths attributed to suicide, homicide or accident, the accidental cases predominated rest of the unnatural deaths. Accidental deaths were more than double the number of homicide, 471/697 (67.5%) versus 219/697 (31.4%). Suicide remained a rarity 7/697 (1.0%). (Table: II) Percentage of urban dwellers was at lower ebb 174 (25%) as compared to inhabitants of peri-urban 314 (45%) and rural areas 209 (30%) ratio remained 5:9:6 (Fig: I). Amongst the total number of accidental deaths (471), 351 persons lost their

lives in road accidents followed by drowning (48) and train track related accidents (42). Road traffic accidents remained the predominant cause 351/471 (74.5%) of accidental fatalities (Table: III). Vehicle occupants (commuters) were found to be victims in 90% of cases and pedestrian's proportion remained low (10%). Amongst homicidal deaths (219) cases, firearm weapons were preferred by the assailants as weapon of assault in 109 (50%) cases followed by traditional conservative weapons, i.e. hard blunt substance 88 (40%) and sharp edged and pointed weapon responsible for killing 22 (10%) cases. The majority of unnatural deaths occurred during summer (April-October) than winter (November to March) ratio being 1.7:1 (Table: IV) The seasonal pattern in this geographical area is hot climate remains for more time (7 months the least) in a year than the relatively cooler months.

Table 1: Gender distribution of unnatural deaths

Year	Males	Females	Total
2006	198 (84.3%)	37 (15.7%)	235 (100.0%)
2007	217 (86.8%)	33 (13.2%)	250 (100.0%)
2008	180 (84.9%)	32 (15.1%)	212 (100.0%)
Total	595 (85.4%)	102 (14.6%)	697 (100.0%)

P-value of Pearson chi-square test is 0.712>0.05

Table 2: Manners of Death in the series

Year	Accidents	Homicides	Suicides	Total
2006	166 (70.6%)	69 (29.4%)	00 (.0%)	235 (100.0%)
2007	178 (71.2%)	69 (27.6%)	03 (1.2%)	250 (100.0%)
2008	127 (59.9%)	81 (38.2%)	04 (1.9%)	212 (100.0%)
Total	471 (67.6%)	219 (31.4%)	07 (1.0%)	697 (100.0%)

P-value of Pearson chi-square test is 0.023<0.05

Table 3: Causes of accidental deaths

Year	Road	Train track	Drowning	Poisoning	Electrocution	Incineration	Total
2006	118 (71.1%)	20 (12.0%)	15 (9.0%)	02 (1.2%)	00 (.0%)	11 (6.6%)	166 (100.0%)
2007	142 (79.8%)	10 (5.6%)	20 (11.2%)	01 (.6%)	05 (2.8%)	00 (.0%)	178 (100.0%)
2008	91 (71.7%)	12 (9.4%)	13 (10.2%)	05 (3.9%)	04 (3.1%)	02 (1.6%)	127 (100.0%)
Total	351 (74.5%)	42 (8.9%)	48 (10.2%)	08 (1.7%)	09 (1.9%)	13 (2.8%)	471 (100.0%)

Table 4: seasonal variation of unnatural deaths

Year	Summer (April-October)	Winter (Nov.-March)	Total
2006	135 (57.4%)	100 (42.6%)	235 (100.0%)
2007	154 (61.6%)	96 (38.4%)	250 (100.0%)
2008	150 (70.8%)	62 (29.2%)	212 (100.0%)
Total	439 (63.0%)	258 (37.0%)	697 (100.0%)

P-value of Pearson chi-square test is 0.012>0.05

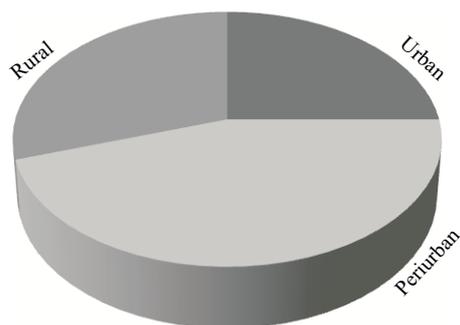


Figure 1: Chart representing the inhabitants of the deceased

DISCUSSION

Catalogue of unnatural deaths includes fatalities caused due to the criminal intent of a perpetrator or result of negligent act or deliberate omission of some person and also those where there is no malafide intent at all but death occurred due to some accident or misadventure.¹⁰

According to census report of 2005, Hyderabad is the 7th populous city of the country inhabiting about 1.4 millions of people.¹¹

Hyderabad is an ideal blend of Urban, peri-urban and rural areas, located at cross roads of three main communicating arteries of the country i.e. Super Highway, National Highway and Indus Highway, on one side it is bounded by River Indus whereas three main water channels of river Indus crosses the city and the suburbs for about 20km distance.

Numbers of unnatural deaths submitted for autopsy each year is low as on the average 232 cases / year are reported. This finding is in contrast to other cities of Pakistan where nearly 600 cases are autopsied per year.¹²

This low number of unnatural deaths can be attributed to comparatively better law and order situation and better intra personal conduct and harmonious relationship amongst the communities in this part of the country. This impression is further strengthened when it is observed that the percentage of homicidal deaths is only 31.4% only in comparison to what has been reported by the other researchers which show a higher rate of homicidal deaths.

Male remained more vulnerable to unnatural death. This observation is in agreement to other authors' findings.¹³ This is because of his work activities, movements, more exposure to outside environments and contacts with other members of society. Over confidence, physical strength, anger, egotism, mindset for revenge are other factors which finally lead him to be the victim or assailant.

Accidents remained predominant cause of unnatural deaths, this finding is also in contrast to the findings of other observers where homicidal deaths occupy more space in the yard stick of unnatural deaths¹⁴ but in agreement with an observer from Karachi who reported that homicidal and accidental deaths are placed evenly.¹⁵

Amongst the accidents, road appeared more dangerous than other means of death because the city is placed at the junction of 3 main roads communicating the economical hub of Karachi with up country. Cargo transportation as well as passengers prefers to travel by roads in Pakistan and vehicular density on the roads leads to fatal accidents, though visibility of roads remains clear throughout the years because of clear weather in this part of the country.

In road related accidents vehicular occupants are the victims in most of the cases than the pedestrians. Majority of the cases die in a vehicular collision or over turning accidents especially the buses and cars. Usually over speeding is reported to be responsible for the casualties.

Majority of the victims are brought from the peri-urban & urban area this is because of the fact that road accidents when occur in the highways, the injured and dead are rushed to the hospital. Liaquat University Hospital being a tertiary care hospital becomes the ultimate choice of the volunteers as well as police to bring the injured / dead to this hospital.

It is not necessary that the dead due to highway accident actually is the inhabitant of that area, but generally recorded in the documents, belonging to the area of the police stations in whose jurisdiction the eventuality occurred.

Rail tract accidents also require further investigations to establish the causes of the incidents whether it is the attributed to the negligence on the part of locomotive drivers, watchman managing the railway crossing or public apathy.

Cases of drowning occupy the 2nd place in the list of the unnatural deaths. This fact is not reported by any other author. The water canals emerging from the nearby barrage traverse the city. People especially teenagers are seen swimming and bathing in the canals especially in the days of summer. There is no restriction to their play and no safety measures are provided for any eventuality, fatality can occur and the funfair ends into funeral.

Suicide remained a rarity like other parts of the country. Shooting for suicide is not seen in any of the case. This is in contrast to the suicidal pattern noticed in USA where suicide by self shooting by males is more common than other means.¹⁶

Hanging remained means of committing suicide in all the cases autopsied amongst the suicidal victim, 5 were males compared to 2 female. This observation is in conformity with gender dominance reported by Indian observers.¹⁷

Gun as a weapon of assault (50%) has outnumbered all other weapons. This fact has become an internationally known fact. In USA more than twenty five thousand people die due to fire arm injury every year.^{18,19} Locally it is documented by various authors that the number of firearms fatalities is of 100% in Dera Ismail Khan and 85.96% in Peshawar.²⁰

CONCLUSION

Majority of victims of un-natural deaths were males dying due to accidental manner of death. peri-urban victims outnumbered rural and urban dwellers. Fatalities were found more in summer season than in winter.

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An In Vitro Evaluation of Apical Microleakage of Single Cone Obturation Versus Lateral Condensation Obturation Technique

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ABSTRACT

Introduction: The aim of this in-vitro study was to use dye penetration method to compare the apical microleakage of matched taper single-cone and cold lateral condensation technique in teeth prepared with ProTaper instruments. **Materials and Methods:** Eighty (80) human extracted single rooted teeth were used. The teeth were randomly divided into two experimental groups of thirty (30) teeth each and two negative and positive control groups of ten (10) teeth each. The groups were as follows: Group I, Teeth were obturated using single cone obturation technique; Group II, Teeth were obturated using a cold lateral condensation technique. In positive control Group; teeth were instrumented and left unobturated, whwre as in negative control Group, teeth were instrumented and five (5) teeth were obturated with single cone obturation technique and five (5) teeth with the Lateral condensation technique. The access cavities of all teeth were obturated with Ketac Molar (3M ESPE) to ensure a coronal seal. The specimens were stored for 24 hours in 100% humidity at 37°C to allow the sealer to set. After that the surface of all roots in experimental and positive control groups were then covered with two layers of nail polish, except for the apical area (2mm). In the negative control group all surfaces of the roots, including the apical area, were covered with two layers of nail polish. Each tooth was subsequently immersed in a freshly prepared 5% aqueous methylene blue dye solution (PH 7.0) at 37°C for seven days, and stored in incubator. Following storage, the roots were cut along their long axis and evaluated under a stereomicroscope to measure the depth of dye penetration.

Results: The negative controls showed no dye penetration while, the positive controls showed completely dye penetration. Mean and standard deviation of leakage for experimental groups were, for Single Cone Obturation, 6.42 (SD ±3.18), for Lateral Condensation Obturation, 6.44 (SD±1.8). There was no significant difference between the two groups (p=0.245).

Conclusion: Both the single cone and the lateral condensation obturation techniques proved equally effective in achieving the apical seal.

Key words: Dye penetration method, Protaper endodontic files, single cone obturation.

INTRODUCTION

The aim of root canal therapy is to attain clean canal that allows for the three-dimensional obturation of root canal system along with a hermetic seal.¹ Obturation provides a seal that prevents reinfection of the canal and subsequent leakage into the periradicular tissues.² It is suggested that incomplete obturation of the root canals (60%) is still one of the major cause of root canal treatment failure.³ Majority of endodontic therapies use gutta perch as root canal

obturation material in several different obturation techniques.⁴ Currently, the most commonly used gutta-percha obturation technique is cold lateral condensation,⁵ and is still the standard with which all other techniques are compared. However, its ability to reproduce the internal surface of root canal has been questioned. Incomplete fusion of gutta-percha cones, voids and lack of surface adaptation has been questioned.⁴

Because of the widespread use of the rotary NiTi systems, manufacturers have produced gutta-percha cones that match the taper of canals prepared with these systems. Preparation of a root canal with rotary NiTi instruments and the use of a sealer with these cones may provide three dimensional obturation of the root canal.⁶ Recently, gutta-percha points for ProTaper (Dentsply Maillefer) have been introduced for simple, time-efficient obturation. In this system, root canals are prepared with the ProTaper instruments and filled with the point that fits the size of the finisher file. The manufacturer claims that ProTaper gutta-percha points perfectly fit canals that have been prepared with ProTaper files.

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Many investigators in their clinical and in-vitro studies concluded that majority of endodontic failures showed incomplete obturation,⁷⁻⁹ which leads to microleakage.¹⁰ Therefore leakage tests are used for the evaluation of the excellence of a root canal filling. A number of scientific articles have been published about various techniques and materials that have been used for achieving a better endodontic obturation with a minimum apical microleakage. In all these studies, it is generally acknowledged that the assessment of the apical leakage of particles or solutions between a root canal filling and the root canal walls is a proper method to establish the quality of an endodontic obturation. The most generally used method for the assessment of apical microleakage is the linear measurement of tracer penetration (e.g. dyes penetration), different concentrations of aqueous solutions of eosin (3, methylene blue,¹¹⁻¹³ or India ink,¹⁴⁻¹⁶ radioisotopes,¹⁷ or bacteria,¹⁸ electro-chemical¹⁹ along the root filling. Measurements were made after the application of different preparation methods, such as longitudinal splitting^{11,13}, cross-sectioning^{12,17}, or decalcification and clearing of the root.¹⁴⁻¹⁶

In spite of the unquestionable improvements, until today no material fulfills all necessities and enviable properties to hermetically seal the root canal system. Apical leakage is still a repeated incident in root-filled teeth, which raises concern regarding the quality of obturation provided by the currently available filling materials.^{20,21} A number of studies have evaluated the apical sealing capability of root canal fillings using different methods.²² Dye penetration method is frequently used to assess leakage due to its simplicity and cost effectiveness.²³

OBJECTIVES

The aim of this in-vitro study was to use a dye penetration method to compare the apical microleakage of matched taper single-cone and cold lateral condensation technique in teeth prepared with ProTaper instruments.

MATERIALS AND METHODS

This prospective experimental interventional study was conducted at the Department of Operative Dentistry and Department of Science of Dental Materials at Dr. Ishrat-ul-Ebad Khan Institute of Oral Health Sciences, (DIEKIOHS) Dow University of Health Sciences, Karachi, Pakistan and testing was carried out at the Department of Materials Engineering at NED University Karachi, Pakistan. The duration of the study was six (6) month.

Eighty (80) single rooted, non carious, unrestored human teeth, extracted for periodontal reasons were selected. Teeth that contain cracks and broken down teeth were excluded from the study. Extracted teeth were taken from Oral and Maxillofacial Surgery Department of (DIEKIOHS).

Extracted teeth were selected, the storage and handling of teeth was conducted according to ISO/TS 11405 standard. All the teeth were radiographed on the buccal and proximal views to check for single root and single canal. Endodontic access was prepared conventionally and the canal system prepared using Protaper system (Dentsply Pakistan) following standard protocol.²⁴ Access cavity was prepared by using diamond round bur, canal orifice was located by using manual canal finder; working lengths were calculated using a size 10 file until its emergence at the apical foramen and then subtracting 2mm. Canal preparation was conducted following crown down technique, coronal third of the canal was prepared by using Sx and S1 files, middle canal was prepared by using S2 and apical portion of the canal was prepared by F1, F2. Sodium hypochlorite (5.25%) was used as an irrigation solution during instrumentation. After final irrigation the canal was subsequently dried with sterile paper points.

The samples were divided into two groups of 30 each specimens to act as experimental groups. Teeth in group I were obturated using single cone obturation technique; a thin coating of the endodontic sealer (Apexit, Ivaclor Vivadent) was manually deposited in the canal using paper point. After that size F2 gutta-percha was coated with Apexit (Ivaclor, Vivadent) sealer, gently seated in the canal. After warming the coronal material was vertically condensed with a endodontic plugger size 4 (Dentsply Maillefer). Teeth in group II were obturated using a cold lateral condensation technique. A size 30 gutta-percha cone was inserted to the working length and a tight fit- assured. The master cone was coated with Apexit (Ivaclor, Vivadent) sealer, gently seated in the canal and condensed with a finger spreader. Accessory gutta-percha cones were inserted until they could not be introduced any further. After warming the coronal material was vertically condensed with an endodontic plugger size 4 (Dentsply Maillefer).

The samples in the control group were divided into two control subgroups of 10 teeth each. Those in the positive control group were instrumented and left unobturated. Those specimens teeth in the negative control group were instrumented and five teeth were obturated with single cone obturation technique and five teeth with the Lateral condensation technique.

The access cavities of all teeth were obturated with Ketac Molar (3M ESPE) to ensure a coronal seal. The specimens were stored for 24 hours in 100% humidity at 37°C to allow the sealer to set. The surface of all roots in experimental and positive control groups were then covered with two layers of nail polish, except for the apical area (2mm). So the tracer could penetrate the canal via the apical region only. In the negative control group all surfaces of the roots, including the apical area, were covered with two layers of nail polish. Each tooth was subsequently immersed in a freshly prepared 5% aqueous methylene blue dye solution (PH 7.0) at 37 °C for seven days, and stored in incubator. A 7-day immersion period in 5% methylene blue was used,

as the procedure did not engage any supplementary active penetration device. In addition, all the canals were prepared to the same final apical size and a same operator conducted both the preparation and obturation on each tooth. After removal from the dye, the specimens were washed with distal water, dried and the nail varnish was removed with scalpel. The root of each tooth was grooved longitudinally on both sides, using a rotating diamond disc under constant cooling with distilled water without disturbing the gutta-percha filling. The roots were sectioned and each half of the roots was examined under stereomicroscope. The amount of leakage was measured in each half of each tooth, from the working length to the most coronal part of the root canal to which the dye had penetrated. Each section was photographed under a stereoscopic microscope (MOTIC, Hong Kong).

STATISTICAL ANALYSIS

Data were analyzed using SPSS version 16. Data were of continuous variables and was normally distributed, statistical descriptive analysis was performed using independent t test to find the statistical difference between the two groups. This enabled the comparison of the apical leakage between the two obturation techniques.

RESULTS

The linear penetration of 5% methylene blue was measured and found to be between 2.12 to 12.21 mm for single cone Obturation and 3.12 to 9.81mm for lateral condensation technique. There were cases where penetration was very small although there were other cases in which a more widespread penetration of 5% methylene blue was observed during microscopic study of serial cross-sections.

The negative controls showed no dye penetration while, the positive controls showed completely dye penetration. Mean and standard deviation of leakage for experimental groups were, for Single Cone Obturation, 6.42mm (±3.18), and for Lateral Condensation Obturation, 6.44 (±1.8%). There was no significant difference between the two groups (p value = 0.245). Positive control groups show 100% leakage while negative control group show no leakage. Dye penetration in the positive control group and absence of dye penetration in the negative control group confirmed the integrity of dye penetration.

Table 1: Mean and standard variation values for the Two Obturation Techniques

Obturation Technique	Sample Size (n)	Mean (mm)	Standard Deviation
Group I Single Cone Obturation	30	6.42	3.18
Group II Lateral Condensation Obturation	30	6.44	1.82

DISCUSSION

Cleaning and shaping play vital in the success of root canal treatment. However, this does not negate the importance of the quality of obturation. This is validated by the fact that nearly 60% of failures in endodontics can be accredited to incomplete obturation of the root canal.²⁵ Hence, a three - dimensional obturation is critical for endodontic success. Irrespective of the obturation technique employed, micro leakage remains to be the most common cause of endodontic failure. Micro leakage is the passage of bacteria, fluids, and chemical substances between the root structure and filling material of any type.²⁶ This occurs because of microscopic gaps at the interface of the filling material and the tooth.²⁷ Microleakage in the root canals is of complex nature as many variables may contribute, such as root filling technique and chemical properties of the sealer and the infectious state of the canal.²⁸

A variety of materials and techniques have been developed to improve the quality of root canal obturations. However, none of these materials and techniques provides a leak proof seal.²⁶ Pashley²⁷ confirmed that microleakage is a severe clinical problem because most dental materials display varying degrees of microleakage. The most significant prerequisites of endodontics are total debridement of the pulpal space, development of a fluid-tight seal at the apical foramen and total obliteration of the root canal.²⁹ Therefore leakage tests are a relevant way to evaluate the apical seal. Methods used to evaluate apical leakage include dye-penetration, electrochemical, radioisotope, bacterial leakage and fluid filtration methods.

Nicholls²⁹ stated that poor seal may lead to voids in the apical region of the canal where Stagnation of tissue fluid can occur. The subsequent proteolysis and irritation can result in persistence of existing periapical lesion or formation of fresh lesions. Traditionally, clinical accent has been on the apical sealing of the root canal obturation. Thus, most leakage experiments^{30,31} have assessed the quality of apical seal by measuring dye penetration in an apico-coronal direction. This technique is simple to carry out but appears to overestimate microleakage i.e. more than the bacteria infiltration method. This might be due to the difference between the sizes of molecules of dyes and bacteria.³²

A dye penetration technique was used in this study to evaluate microleakge between two different obturation techniques by microscopic observation (stereomicroscope). Lateral condensation is a generally accepted technique and it was compared to a single cone obturation method. Methylene blue is a small molecular weight dye which has high penetration ability.³² It is highly susceptible to demineralization and thus can lead to observation bias in clearing techniques.



Fig 1. Initial Filing and working length assessment

Fig 2. Drying of canal with sterile paper point

Fig 3. Single cone obturation

Fig 4. Marking of apical 2mm of root portion

Fig 5. Tooth samples coated with nail varnish

Fig 6. Sample placed in 5% methylene blue dye

In recent years, rotary nickel-titanium (NiTi) instruments have become popular because of their superiority over stainless steel hand files, elasticity and resistance to torsional fracture³³. Moreover, rotary NiTi instruments improve working safety, shorten working time and prepare well-shaped root canals with fewer canal transportations^{34,35}. Recently, new file designs of rotary NiTi instruments with sharp cutting edges called ProTaper (Dentsply Maillefer, Ballaigues, Switzerland) were introduced. The ProTaper system features just 6 instruments: SX shaper, 2 shaping files (S1 and S2) and 3 finishing files (F1, F2 and F3)^{36,37}. This system work with multiple obturation techniques such as single cone obturation.

The single-cone technique consists of a single gutta-percha cone filled at room temperature with sealer layer thicknesses that vary, depending on the adaptation of the single cone to the walls of the canal.³⁸ Single-cone obturation has not been well regarded because of the use of large amounts of sealer. Porosities in large volumes of sealer, setting contraction and dissolution of the sealer are the main disadvantages of this technique.³⁹ The poor seal of the material because of shrinkage after setting is a significant problem.⁴⁰

In the literature it has been reported that obturations of lateral condensation had a better treatment outcome than single cone obturations.⁴¹ However, these obturations were done with standardized .02 taper gutta-percha cones, usually with zinc-oxide-eugenol-based sealers. Because large volumes of this soluble sealer were used, dissolution of the sealer leading to microleakage may have had a negative effect on the outcome.³⁷ In this study, Apexit sealer, a Calcium hydroxide-based, radiopaque root canal sealer was chosen because of less shrinkage for the final endodontic Obturation.

Pommel and Camps⁴² compared single-cone, lateral condensation, vertical condensation, Thermafil and System B techniques using a zinc-oxide-eugenol-based sealer and reported that the single-cone technique had the highest leakage. On the other hand, Wu and colleagues³⁸ studied the leakage of single-cone fillings using a silicone-based sealer for 1 year and concluded that single-cone fillings prevented fluid transport for 1 year.

With the widespread use of rotary NiTi instruments, matched-taper gutta-percha cones were developed and the single-cone technique has become popular again. Gordon and colleagues⁴³ reported that the cross-sectional area of the .06 taper single-cone technique was comparable with

that of lateral condensation, and the taper single-cone technique was faster than lateral condensation. Bal and colleagues⁴⁴ compared the sealing ability of root canals prepared with .06 rotary NiTi instruments and obturated with either a .06 or a .02 tapered gutta-percha master cone using lateral condensation and found no difference between the groups.

More recently, Zmener and colleagues⁴⁵ prepared the root canals using a rotary system and obturated with single-cone and lateral condensation techniques. They reported that the difference between single-cone and lateral condensation obturation was not significant.

In our study the results showed no significant difference between the two groups and the extent of microleakage in the single cone obturation technique was almost equal to Lateral Condensation technique ($p=0.245$). This was in agreement with the results of Bal and colleague,⁴⁴ Zmener and colleagues⁴⁵ and Gordon and colleagues.⁴³ However Pommel and Camps⁴² found the opposite results, which may be because of difference in the sealer.

Wu and Wesselink³⁸ concluded that data obtained from the linear measurements of dye penetration after longitudinal splitting or decalcification and clearing of roots varied to a much higher extent than the data obtained after cross-sectioning of the samples.

In this study the dye penetration method only provide qualitative data of linear measurement of tracer penetration along the root filling and did not provide any information about the volume of the tracer that penetrated along the root canal obturation, However, the teeth used in this study had single straight canals but posterior teeth have narrow and curved canals with complex anatomy, which might present greater challenges hence are the limitations of the study. Further study is needed to evaluate the sealing ability of obturation of matched-taper gutta-percha cones to determine whether these obturations have an acceptable apical seal.

CONCLUSION

In the current study, the results of matched-taper single-cone obturation were compared with lateral condensation technique and it was concluded that there was no significant difference in efficacy of apical seal achievement between the two groups. However further studies are required to confirm this relationship.



Fig 7. Incubator

Fig 8. Tooth sample after 7days storage in incubator

Fig 9. Scraping of nail varnish

Fig 10. Longitudinal sectioning of tooth with diamond disc

Fig 11. Microleakage assessment under Stereomicroscope

Fig 12. Microleakage assessment with naked eye

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To Evaluate the Effect of Shelf life on Depth of Cure of Polyacid-Modified composite – An In Vitro Study

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ABSTRACT

Objective: The present study aimed to compare the curing depth of chemically similar Polyacid-modified composite resins (PAM-C) having different expiry periods.

Methods: The curing depth of the PAM-C, Dyract Extra (Dentsply, U.K.) was determined for a near expiry and a long expiry material using a scraping method based on ISO 4049:2000. Samples were light-cured (800 mW/cm² at 40 seconds) in plastic mould. Immediately after light-curing the cylinder shaped material was removed from the mould, height of the cylinder of cured material was measured by using digital caliper and taken as the curing depth. The means of the curing depth of two materials were subjected to two sample independent t test using SPSS.

Results: The mean value of depth of cure for near expiry PAM-C (Group-A) was 6.389 mm (sd ± .202) and that of long expiry material PAM-C (Group-B) was 7.087 mm (sd ± .149). The curing depth differed significantly between the materials of the two groups (P<0.001).

Conclusion: The curing depth greatly varies between the materials. It may be inferred that the curing depth of the two assigned groups of PAM-Cs depend on the period of expiry of the material.

Key words: Shelf life, Depth of cure, Polyacid modified composite.

INTRODUCTION

Polyacid-modified composites were devised by combining the aesthetics of traditional composite resins and fluoride releasing ability of GIC cements. These materials were an amalgamation of “Compo” meaning composites and “omer” from glass-ionomer cements.¹

Compomers like traditional composites contain resin phase containing BISGMA or UDMA diluted with TEGDMA, an inorganic non reactive filler phase of quartz, aluminosilicate glass or SrAlFSiO₄ particles along with a photoinitiator system.² A silane coupling agent is used to create a bond between the filler and the resin phase.³

The differentiating factor between compomers and traditional composites is the presence of additional monomers TGB with acidic functional groups. Due to this modification compomers are also referred to as polyacid-modified composites.⁴

They set by polymerization reaction of the acidic monomers since they contain camphorquinone as an initiator and amine as the accelerator. On exposure to radiation energy released from the lamps, the initiators form free radicals and addition polymerization reaction commences to form polymers.⁵

Historically, UV lamps were used to photo-polymerize the composites. Because of their limited ability to penetrate deep within the material, harmful effects on human eye and possible changes in oral microflora, UV lamps have been replaced by visible light lamps.⁶

Contemporary curing lamps used to cure resin based restorative materials are halogen lamps, LED lamps and plasma arc curing lamps. The amount of radiation reaching a certain depth in a material may depend on the factors such as lamp output intensity, exposure time, distance from light source to material and curing depth.⁷

LED lamps have gained popularity over low intensity halogen lamps due to their ability to cure compomers at greater depths.⁸

The contributing factors in curing depth of resin composites are chemical composition and shade of resin composite, light intensity and wavelength.⁹

Compomers like composites are hydrophobic but also imbibe water and promote secondary acid base reaction.¹⁰

As far as polymerization is concerned, compomers also have similar problems as those of dental composites i.e. limited depth of cure and formation of contraction stresses.¹¹

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These stresses can be reduced by the uptake of water, however, depth of cure of compomers vary widely depending on the brand and shade of compomers used. Koupis using scraping method and the penetrometer demonstrated that the curing depths of compomers can be smaller than the microfilled resin composite. He concluded that compomers with shade A-2 have greater values of depth of cure as compared to other shades. 2 mm incremental placement of the material to the cavity is also advised.¹²

It is a common practice but not reported in any literature that some dental professionals in local settings prefer to buy and use materials having very short expiry as these are available at cheaper prices. The present study was performed to evaluate whether this practice would make any difference in polymerization of the material when it is compared with a material having a long expiry date.

The aim of the present study was to evaluate the effect of shelf life on the depth of cure of polyacid-modified composites.

The null hypothesis for this study was that the depth of cure of PAM-C did not differ for two materials having short and long expiry times.

MATERIALS AND METHODS

The experimental study was conducted at the Operative Dentistry Department and Science of Dental Materials Lab of Dr. Ishrat ul Ebad Institute of Oral Health Sciences, Dow University of Health Sciences, Karachi, Pakistan.

The material under investigation in the present study consists of Urethane dimethacrylate (UDM), TCB resin Tri ethylene Glycidyl dimethacrylate (TEGDMA), camphorquinone UV stabilizers, Strontium-alumino-sodium-fluoro-phosphor-silicate glass, highly dispersed silicon dioxide, Strontium fluoride, Iron oxide and titanium oxide pigments.

Sample size

Sixty cylinder shaped specimens, divided into two groups i.e. Group A (n=30) comprising of specimens having near expiry date and Group B (n=30) comprising of specimens having long expiry date.

The depth of cure of the materials was determined according to ISO 4049:2009. Compomer of the each group was filled into a plastic mould (Dentsply - Caulk, UK) of dimension 4mm X 8mm. The excess material was removed by pressing a glass slide against the top of the plastic mould. The material was light cured using a LED light (Hilux) for 40 seconds and a 0.5 mm distance from the tip of the curing light to the upper surface of the filled mould was maintained. The power density of the light was checked with a radiometer before every cure and was maintained at 800 mW/cm². 30 samples were prepared for each testing group i.e. one group

having Dyract Extra PAM-C (Dentsply – Caulk , UK) of near expiry date (One week from expiry) and another group consisting of the same material having a long expiry date (2 years from expiry).

Each cylinder shaped specimen of the cured material was removed from the mould and the soft un-polymerized material was scraped off using a plastic spatula. The length of the remaining material was measured by using a digital caliper (Nakamura Mfg. Co., Ltd, Tokyo, Japan) and recorded as the curing depth. The samples having fissures or curing defects were excluded and new specimens with perfect dimensions were prepared to be included.

Statistical Analysis

The data were entered in the spss version 16. Two samples t test with 95% confidence interval was used to determine statistical difference between the mean values of the near expiry and long expiry PAM-C.

RESULTS

According to Table 1. The mean value of depth of cure for near expiry PAM-C was 6.389 mm (± 0.202) and that of long expiry material PAM-C was 7.087 mm (± 0.149). The data suggest that PAM-C with long expiry has, on average, a depth of cure more than that of PAM-C with near expiry (Table 1).

The data provide strong evidence of a difference (0.698mm) between the means of near and long expiry PAM-C, the P value came out to be <0.001 (Table 1).

Table 1: Descriptive statistics of depth of cure of near and long expiry PAM-Cs

Expiry Status	N	Mean	Std. Deviation	P-value
Near Expiry	30	6.3890	20244	<0.0001
Long Expiry	30	7.0870	14972	<0.0001

DISCUSSION

In compomers, a number of factors are responsible for the adequacy of their polymerization process. Factors that are important are the type of filler used, the initiator /catalyst system and the most important is the type and amount of monomer used in the composition.¹³

This study evaluated the effect of shelf life on the adequacy of the polymerization process and its effect on the depth of cure of compomers.

The presence and post-cure release of residual monomers can have a negative impact on the biocompatibility of the material. In order to minimize these harmful effect halogen curing lights with a power density of atleast 250-300mW/cm² should be used.¹⁴

Polyacid modified composites like traditional composites have a limited depth of cure and their composition consists of additional monomers with acidic functional groups and degradable alumino-silicate glass particles.¹⁵

The depth of cure for compomers is controlled to a large extent on the opacity of the glass particles used. Thus the formulation of compomers plays an important role in determining the depth of cure, however literature states that lighter shades of compomers cure to a greater depth than do darker shades. In comparison with microfilled composites compomers cure to lesser depth depending on their shade.¹⁶

This study considered the effect of the shelf life on the depth of the cure of compomers with a specific formulation. It was observed that there is a significant difference in the depth of cure of the two chemically similar compomers. The compomer that had a long expiry date had a greater value of depth of cure than the compomer that had a short expiry date. It could be hypothesized that unexpired compomers had more amount of residual monomer in their matrix that matured over a period of time due to post cure, resulting in greater depth of cure. Whereas the compomer that had a short expiry date had less residual monomers, which resulted in lesser depth of cure. However no concrete correlation could be determined between the compomers with the different expiry dates and their depth of cure value. It must also be noted that light absorption and light scattering in resins reduces the degree of monomer to polymer conversion hence could have some effect on the depth of cure of the compomers with differing expiry dates.

Increasing curing lamp intensities and reducing the exposure time can also lead to an increase in the depth of cure of the compomers.¹⁷

Dunne has concluded in his study that depth of cure of resin is directly related to intensity of the curing light, duration of light exposure and inversely proportional to the distance of the light source.¹⁸

Depth of cure was also evaluated among different shades of resin composites and it was documented that shade A2 results in greater values of depth of cure than shade A4, the effect depending quantitatively on the formulation of the material. A layer of 2 mm is recommended to be applied in the incremental technique but in this study some formulations of PAM-Cs do not reach a depth of cure of 2 mm. Therefore a hard top surface of a PAM-C is not an indication of adequacy in depth of polymerization.^{19,20}

CONCLUSION

The results observed in this study suggest that a greater depth of cure can be achieved with compomers having a long expiry as compared to compomers having a short expiry date. More experiments need to be carried out to find a relation between the value of depth of cure and expiry date.

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Frequency of Risk Factors Associated with Road Traffic Accidents of Motorbike in a Big City of a Developing Country

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ABSTRACT

Objective: To determine the frequency of risk factors associated with road traffic accidents of motorbike in Karachi, Pakistan.

Methods: A descriptive cross-sectional study was carried out in the two tertiary care hospitals (Civil Hospital and Jinnah Postgraduate Medical Center) of Karachi, Pakistan. All male motorbike accident victims presented to the emergency department of the selected hospital, who gave consent, were included in the study. Patients under age 15 years and do not understand Urdu language were excluded. Information was collected through a pretested questionnaire.

Results: A total of 127 accident cases from respected hospitals were included in the study. Most (76%) of the accident cases were younger than 33 years. Approximately, 49% of the participants were riding the bikes without driving license. Nearly, 43% never used helmet during riding. Approximately, 21% were reported that they were chewing tobacco at the time of accident. Majority (71%) of the accidents occurred on the working days. Almost 61% of the accidents occurred in the evening time. In 43% of the cases, speed of the motorbikes was more than 50 km/hour. Nearly, 31% of the victims were riding along with their friends/family members. Majority (72%) of the accidents occurred on the main roads. Approximately 45 % of the accident cases had fracture. Approximately, 34% of the injuries were severe and lower extremity was involved in 36% of cases. About, 38 % of the victims reported that they were in depressed moods while riding bike.

Conclusion: Mental depression, young age and lack of driving expertise were the major risk factors for motor bike accidents.

Key words: Motorbike accidents, road traffic accident, accidents in Pakistan.

INTRODUCTION

Road traffic accidents (RTAs) have been the major contributor to the global burden of disease and it is predicted that RTAs will be the third leading cause of death by 2020.¹ Death from RTA account for around 23% of all deaths from injuries and 2.1% of all deaths globally. Annual numbers of deaths from RTAs range from 750,000 to 1183,492 (approximately 3000 deaths/day on roads throughout the world) and 10/1000 become disable for life.² Injuries result in major financial and productivity losses to nations along with imposing an astonishing impact on individuals and their families.^{3,4}

However impact of road traffic injuries is far greater in developing countries as compared to developed countries.⁵

In Pakistan, injuries are fifth leading cause of loss of healthy life, and second leading cause of disability.⁶ Death rate from RTA is 4 per 100,000 population and 15 per 10,000 vehicles in Pakistan.⁷ Non-fatal injuries of 19 per 10,000

population has been reported from Karachi.⁸ Total number of registered vehicles increased 17 times from 1956 to 1996 and during this period there were a 5 fold increase in number of vehicles on roads.^{3,9} There was a 14 fold increase in accidents and similarly deaths following RTAs increased 16 times during this time in Pakistan.^{3,9}

In RTAs, the cyclist, motorbike riders and scooter riders are mainly the victims and suffered from major injuries and consequently deaths.^{10,11} Motorbike ride needs quick decisions as one has to respond immediately to stop or turn around in case of coarse road.^{10,12} Increase in motorcycle and scooter use in recent years has increased deaths and injuries in motor bike users.^{11,13} The Motorbike riders are more prone towards injuries of head, chest and extremities.^{10,14,15} The nature of wound, fractures, dislocation of the limbs, thoracic injury and head injury is usually severe in case of motor bike accident as compared to other accident cases.¹⁴ Studies reported that majority of injuries related to motorbike riding occur in young people, who generally tend to adopt risky attitudes and behaviors.^{10,14,15} The risk of accident depends on many factors such as rider's age, gender, experience, motorcycle mileage, attitude and behavior of the rider, license, helmet use, alcohol use, type and conditions of road.^{10,12,15} The main reasons for increasing RTAs in this region is continuously increase in number of motor vehicle, poor enforcement of traffic safety regulation, poor quality of roads and vehicles and inadequate public health infrastructure.^{10,12,16} There is a lack of reliable data on injuries in developing countries, because it is primarily recorded by hospital and police authorities to be used for

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legal purpose and comparison of police record and hospital data on injuries shown that police data is under reported.^{3,11,13,17,18}

Road traffic injuries related to motorbike accidents are major but neglected global public health problem in our part of the world, which needs immediate efforts to deal with for prevention of RTAs on long term basis.^{16,17} In order to understand the reasons for the increasing number of motorbike accidents we need to explore the road conditions, rider's skills, experience and attitude, which need to be targeted in order to reduce the burden of motorbike accidents. Identification of associated factors of motorbike accidents which are modifiable, culturally appropriate and cost effective can help in reducing the burden on these accidents on health care.^{10,19} Findings of our study will be helpful in making the basis for initiation of primary prevention programs to control the epidemic of RTAs in our set up. The objective of this study was to explore and quantify the risk factors related to motorbike accidents in Karachi, Pakistan.

METHODOLOGY

This is a cross sectional study, conducted in two tertiary care hospitals of public sector (Civil Hospital and Jinnah Postgraduate Medical Center) in Karachi, which deal with a major bulk of accident cases in city.²⁰ All motorbike accident victims presented to the emergency department of the selected hospitals fulfilling eligibility criteria (male, willingness to participate) were included. Motorbike injury cases under age 15 years and those who don't understand Urdu language were excluded. A total of 127 motorbike accident patients were included in the study during August 2008 to October 2008.

The information on demographic, social, motorbike and accident related variables such as individual attitude of rider, personal characteristics and experience level was gathered through a questionnaire. Pre-testing of the studied on 15 subjects to assess its appropriateness. Informed oral consent was taken from each participant after explaining the study purpose. Data collection was carried out through trained data collectors. Principal investigator was actively involved in monitoring data collection process to assure the data quality. During training, data collectors were explained about study objectives, methods and sampling technique, proper way of conducting an interview, maintaining the confidentiality of the study subjects and correct way of filling questionnaire. They were also trained about the field editing of the filled questionnaire.

Data were entered through software the Epi-Info version 3.1 and initial data entry file was then transferred into SPSS file for data analysis. Only descriptive statistics were calculated for all the variables in the study, which includes calculation of proportions for categorical variables like age categories, bike riding history, helmet use, day and time of accident and road condition, site of accident and severity of injury.

RESULTS

Data were collected on total of 127 motorbike accident cases. Table 1 summarizes the characteristics of the motorbike injury cases and the traffic conditions associated with motorbike accidents.

Approximately 16% of the motor bike injury patients were younger than 19 years, 60 % were between 19 to 33 years and 24% were more than 33 years old. About 8% had less than 1 year experience of bike riding, 38% had 2-5 years' experience, 25% had 6-10 years' experience and 29% had more than 10 years' experience of bike riding. Approximately, 49% of the injury cases were riding the bikes without having license. Around 43 % of the injured cases reported that they never used a helmet during bike riding.

Table 1: Characteristics of the motorbike riders suffered from motorbike accident and general road traffic conditions related to motorbike accident

Variables	n	%
Age (Years)		
Less than 19	20	15.7
Between 19 to 33	36	59.9
More than 33	31	24.4
Bike riding experience		
Less than 1 year	10	7.9
Between 2-5 years	48	37.8
Between 6-10 years	32	25.2
More than 10 years	37	29.1
Average bike riding (km/day)		
Less than 10	26	21.1
Between 11-20	20	16.3
More than 20	77	62.6
Motorbike License		
Yes	67	51.2
No	59	48.8
Habit of wearing helmet		
Always	25	19.7
Sometimes	47	37.0
Never	55	43.3
Day of the accident		
Working days	89	71
Weekends	36	29
Time of accident		
Day time	49	39
Evening time	78	61
Type of the road (at accident time)		
Main road	92	72.4
Small lane	21	16.5
Round about	14	11.0
Pattern of the road (at accident time)		
One way	72	75.1
Two way	42	33.3
Round about	12	9.5
Source of collision		
Slipped	25	21.4
Car	44	37.6
Bus	15	12.8
Coach	11	9.4
Truck	5	4.3
Rickshaw	4	3.4
Bike	13	11.1
Cause of the accident		
Mechanical fault	14	11.4
Ride error	44	35.8
Wet road	15	12.2
High speed	19	15.4
Opponent mistake	26	21.1
Rider error	5	4.1
On the wrong way		
Bike rider	27	26.5
Opponent	75	73.5

Majority (71%) of the accidents occurred on working days. Similarly, 61% of the accidents occurred in evening time. Most(72%) of the accidents occurred on main roads, while 17% occurred on small lanes and 11% near a roundabout. Reported reasons by motorbike riders for motorbike accidents were; rider’s personal mistake (36%), high speed (15%), mechanical faults (11%) and opponent’s mistake (21%). Opponent is defined as the person (other than the injured participant) on the other vehicle with whom collision has occurred at the time of accident. Approximately, 74% reported that their opponent was on the wrong side at the time of the motorbike accident. Source of collision was a car in 38% of the accident cases. Road user behaviors associated to motor bike accidents are given in table 2. Speed at the time of the accident was more than 50 km/hour in 43% of the accident cases, between 31-50 km/hour in 36% and less than 31 km/hour in only in 21% of the accident cases. Most (69%) of the motorbike riders were alone at the time of the accident. Only 4% reported that they broke the signal at time of accident. Almost 72% reported that they were not wearing helmet at the time of accident. Approximately 20% reported that they were talking at the time of accident with person with whom they were riding, 21% were chewing tobacco during bike riding and 38% reported they were in depressed mood at the time of accident.

Table 3 describes patterns of injury, type and severity in motorbike riders. Nearly, 45% of the victims got bone fracture. Approximately, 29% of the injuries were mild, 38% were moderate, and 34% were severe. Injuries are divided into mild, moderated and severe form on the basis of Injury Severity Score (ISS) of Abbreviated Injury Scale (AIS) as used by the participant hospitals. Upper extremity was involved in 30% cases, lower extremity was involved in 35% of the injury cases, head injured in 20% of the cases, chest and ribs in 10% and back in 5% of the case.

DISCUSSION

Motorcycle accidents have somewhat different characteristics when compared with other vehicles group. Motor cyclists themselves seem to have far more problems with other types of accidents, such as those on bends/turns and over taking accidents etc. Our findings suggest that most of the accident cases occur in bike riders who are under age of 30 years. There is a possibility that this trend occurred because young age group is over represented in our sample cases but our findings are consistent with other studies that young motor bike riders most likely to experience an accident because of deliberate risk taking by the driver such as non use of helmet and high speed.¹¹ One study from Singapore by Tham et al was conducted to see the pattern of injuries in helmeted motorbike riders also reported that mean age of motorbike accident case was 32.5 years which was significantly lower than other vehicle accident patients.¹⁴

Table 2: Attitude and behaviors of motorbike riders suffered from motorbike accidents

Variables	n	%
Speed at the time of the accident		
< 30 km/h	26	20.5
31-50 km/h	46	36.2
>50 km/h	55	43.3
Number of persons on bike		
One	88	69.3
Two	32	25.2
Three	7	5.5
Break the signal		
No	121	95.3
Yes	6	4.7
Wearing helmet		
No	88	72.1
Yes	34	27.9
Talking with other person		
No	102	80.3
Yes	25	19.7
Looking at the sign board		
No	112	88.2
Yes	15	11.8
Attending a call		
No	126	99.2
Yes	1	0.8
Listening music		
No	120	94.5
Yes	7	5.5
Depressed mood		
No	78	61.9
Yes	48	38.1
Smoking		
No	119	93.7
Yes	8	6.3
Chewing tobacco		
No	99	78.6
Yes	27	21.4

Table 3: Patterns and severity of injuries resulted from accident among motorbike riders

Variables	n	%
Bone injury		
No	69	54.8
Yes	57	45.2
Severity of injury		
Mild	36	28.8
Moderate	47	37.6
Severe	42	33.6
Site of injury		
Upper extremity	53	29.9
Lower extremity	63	35.6
Head	35	19.8
Back	9	5.1
Chest / Ribs	17	9.6
Bone involved in injury		
Wrist	4	5.3
Ulna	3	3.9
Tibia	10	13.2
Skull	4	5.3
Femur	21	27.6
Radius	6	7.9
Ankle	2	2.6
Hip	3	3.9
Ribs	6	7.9
Knee	4	5.3
Fibula	7	9.2
Shoulder	5	6.6
Metatarsal	1	1.3

A hospital based study from Nepal highlighted that the majority motorbike accident victims were aged between the years 16-30.²¹ Similarly data collected at New south Wales Road by traffic authority between 1996-2000 showed that more than 80% of the victims were in the age group between 14-15years.²² A cross sectional study of fatal accidents in Colorado revealed that learner driver-aged 16 reported to drive four times more carelessly and three times more traffic laws violation were reported as compared to older age range between 25-49 years drivers group.²³ Another study from California reported that according to a police crash database between the years 1993-1996 teenager bike riders, driving in night had been noted to have a high risk of accidents.²⁴ Clarke et al also reported that most of the motorbike accident occurred in the evening time, our results are also showing the same trends because in evening time roads are too busy with all type of vehicles, increases the risk of accidents.¹¹ Our findings are also supported by another study that reported that fewer accidents occur on weekends as compare to work days.¹¹

Studies report that along with culture and socioeconomic status, there are differences in constellation of risk factors contributing to road traffic crashes in urban and rural areas as well.^{25, 26} Our data also suggested that there are certain road conditions which making motorbike riders prone towards accident i.e. slippery (wet) roads, roundabout and small lanes. In our study, approximately 73% of cases reported that it was mistake of opponent which led to accident. Our finding regarding opponent mistake is also supported by Clarke's study in which 65% of the cases reported opponent's mistake as a reason for accident and mostly car drivers were the opponent in accidents.¹¹ A study from Tehran, which was conducted to see the pattern of motorbike injuries reported that in most of the crashes it was opponent's mistake which was responsible for accidents.¹⁸

There are certain other factors besides experience of driving which are responsible for higher risk of accident like high speed and lack of knowledge of traffic laws. The specific behavior and carelessness was assessed through responses about listening music, talking with another person, looking at sign boards, helmet use and number of persons on the bike. Experience of motor bike rider is difficult to quantify and it is reported that most of the injuries are concentrated in inattentive bike riders.¹⁰

In Pakistan there is a major issue of insufficient knowledge of traffic rules and the importance of use of helmets during motorbike riding.²⁷ This fact is evident in our study as 72% of the cases were not wearing a helmet at the time of accident where as in a study by Zargar et al conducted in Tehran, 91.4% of the cases reported that they were not wearing a helmet¹⁸. Studies have reported that severe injuries are more in cases who don't use a helmet.^{15,16}

High speed was also identified as one factor associated with motorbike injury which is consistent with other studies.^{10,11} A cohort study in Sweden conducted to see the factors associated with motorcycle accidents reported that alcohol consumption, driving in rural area and speeding over 50 km/hour were increasing the risk motorbike accidents in the young bike riders.²⁸ Jooma et al, in his survey on head injury reported that more severe injuries occur in patients through RTA as compared to other injuries.²⁰

Wound, fractures and dislocation of the limbs and severe thoracic injury were also more prevalent in motor bike accident cases as compared to other accident cases as reported in many studies and our data also showed that injuries to the extremity and head injury is more common in bike riders as compare to other form of accidents.^{18,29} Among motorbike rider with head injury, more than one third had severe head injury in Tham's study.¹⁴ Adherence to motor cycle helmet law has significantly reduced severe injuries and fatality in many countries of the world.^{16,30,31}

There are certain limitations to our study. This is a hospital based study and we are taking only accident cases and do not included information of healthy participants so we can not estimate incidences of motorbike accidents in the community. A community based study would add more in knowledge about incidence and type of injuries in motor bike accidents. Our study does not include data about patients who died before reaching to hospital because of severe motor bike injury. Power of our study might be low due to small sample size, so it may not reflect the actual magnitude of the motorbike accidents.

Motor bike accident can make a victim permanently disabled. Together with pain and suffering, RTA indulges family into a very huge economic burden. The trauma and economic loss to families and employers expands beyond the motorbike accident victim into the community.⁴ Implementation of helmet law will increase helmet use and decrease fatalities and injuries among motorcyclists.

CONCLUSION

Young motorbike riders and those with habits of using tobacco during bike riding (responsible for diverting attention during riding) and those who ride motorbike without license are at greatest risk of motorbike accidents. Initiatives for motorbike rider's safety should address the behavior of the both motorbike rider and the other road users as well. The education should be given about the driving habits, speeding, safe distance, driving conditions, and license. Both skill and attitude of the rider needs to be focused.

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Microcephaly: Tracing the Evolutionary Lineage of ASPM gene

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ABSTRACT

Objective: Microcephaly, in the form of congenital autosomal recessive disorder (MCPH), is characterized by the reduced occipital frontal head circumference >3 standard deviation of otherwise normal population of matching age and sex. The disease is primarily associated with mild to severe mental retardation. Earlier studies have unravelled that among Pakistani population, mutations in *ASPM* gene is strongly associated in MCPH. In the present study, we have explored the ancestral root of this disease and the process involved in its evolution using tools of bioinformatics.

Experimental Methods: cDNA gene and protein sequences of *ASPM* gene were retrieved from NCBI database and subjected to the non-redundant BLAST. Consensus phylogenetic tree was developed after multiple sequence alignment and bootstrapping of the protein sequences of *ASPM* gene from different mammals using Neighbour Joining method, selecting non mammals as an out group. Comparisons of the gene synteny and exon and intron patterns of *ASPM* gene were also undertaken to investigate chromosomal changes during the course of human evolution. Different statistical evolutionary models namely, Codon Based Z test and Maximum Composite Likelihood Estimate were used in order to estimate the nature of nucleotide substitution and the type of selection pressure the gene has undergone.

Results: Phylogenetic tree based on *ASPM* gene clearly segregated all non mammalian members as an out group. Mammalian in group holds the established evolutionary lineage, based on morpho-genetic attributes of mammalian evolution, segregating monotremes at the beginning followed by the members of rodentia and finally radiation of the primates including humans. Orientation of the *ASPM* gene remains conserved between human and chimpanzee, however, it was found reversed along with two flanking genes, a zinc finger binding domain 41 and coagulation factor XIII, which suggest relatively recent event of gene inversion. Some earlier and, in comparison, more intricate chromosomal changes have also been detected among the lower order of mammals. Aligning *ASPM* gene exons with the primates and lower order mammals indicates transitional bias of mutation over transversion (R value= 1.563). Holistically, codon based Z test revealed positive selection pressure on of *ASPM* gene from rodentia to primates.

Conclusion: Briefly, the studies highlights the evolutionary events of *ASPM* gene in mammals especially primates including humans. Further studies in connection to correlating the cranial cavity size and ancestral gene sequences and in depth sequence comparison would be more insightful in this regard and studies in this connection are ongoing and will be reported shortly.

Key words: Microcephaly, Human Evolution, ASPM gene.

INTRODUCTION

Human brain, in its normal physiological capacity, is arguably the most fascinating milestone achieved during the course of human evolution. The notion is self evident if one compare the sheer intellectual and communication fire power and in many instances dexterity of the humans with other closely and distantly related organisms. Increased size of the brain in comparison to body (encephalization)¹

is the most important attribute in this regard and any anomaly that leads to the reduction of the brain size is responsible for the partial and/or complete loss of such distinctive characteristics. One such illness is called as microcephaly. Microcephaly (MC) is defined as a significant reduction in head circumference (>3 SD below the mean) for an individual's age and sex.² It is a multifactorial diseases which can either present itself as a single disease or as part of a syndrome. Dianne Abuelo has previously reviewed various symptoms and disease associations of MC in detail.³ Several environmental and genetic factors have been found responsible for the microcephaly. Common environmental factors that cause MC include antenatal exposure to teratogens including alcohol, hydantoin and radiation amongst others. Genetically, the disease may present as an autosomal dominant, autosomal recessive or X-linked traits.³

On the basis of its onset, the disease is broadly classified into primary MC, which arises due to the impairment of the brain development during gestation period, and secondary MC, which develop during the postnatal period.⁴

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Autosomal recessive primary microcephaly (MCPH) is a subtype of primary MC in which reduction of head circumference is associated with mild to severe mental retardation and/or other neurological symptoms such as seizures cognitive deficits etc.⁵ Alarmingly, compared to 1 in a million in British population, incidence of MCPH in Pakistan is 1 in 10,000.⁶ More recently various genes have been found associated with the occurrence of MCPH. These genetic loci include *MCPH1*, *MCPH3*, *MCPH5/ASPM* and *MCPH6*.^{7,8,9,10,11} Amongst all the mutations identified in different microcephaly associated genes, *ASPM* mutations have been reported to be the most common cause of MCPH in Pakistan.^{12,13}

ASPM (Abnormal spindle like microcephaly-associated protein) gene encodes for a protein which expressed in the cerebral cortical ventricular and proliferative zones of medial and lateral ganglionic eminences during neurogenesis. Postnatally, *ASPM* also expresses in the regions of constant neurogenesis. The gene regulates the normal mitotic spindle function in the embryonic neuroblasts and therefore it is required for proliferation of neuronal cells to attain the required brain size. Mutations in the genes generally results in the production of truncated non functional protein which impairs the normal brain development and causing microcephalic conditions.^{14,15}

Several studies have been conducted which relates the sequential transitions of *ASPM* gene in different mammals with their brain size.^{6,14,15,16} However, the nature of selection pressure and transformation of genomic synteny underlying the very process of *ASPM* evolution is still debatable or largely unaddressed. In the present study we have explored the ancestral lineage of *ASPM* genes among different representative mammals including human. The study has been further extended to investigate the changes which occurred in the *ASPM* gene order of the compared organism while going through evolution. Sequences were also analysed using suitable mathematical and/or statistical models to unravel the nature of selection pressure in this connection. It is our belief that the present study will provide more insights to the evolutionary history of microcephaly with reference to *ASPM* gene. To the best of our cognizance the study is the first report to illustrate the genomic architectural changes of *ASPM* during its evolution.

METHODS

Sequence Analysis and Evolutionary Tree Construction

Protein and cDNA (CCDS 1389.1) sequences of Human *ASPM* gene were retrieved from dBest database of the NCBI (National Centre for Biotechnology Information).¹⁷ The sequences were subjected to non-redundant BLAST (Basic Local Alignment Search Tool) in FASTA format.^{18,19}

The homologues of the *ASPM* were retrieved and multiple sequence alignment were generated using default parameters (with some manual adjustment) of program Clustal X and MEGA4 (Molecular Evolutionary Genetic Analysis version.4).^{20,21} The consensus boot strapped (500 replicas) phylogenetic tree was constructed from *ASPM* protein sequences using Neighbour Joining method.

Genomic Organization

Information regarding *ASPM* and its flanking genes organization of selected animals were taken from NCBI gene database. For the sake of comprehensibility, the schematic representation were developed using Microsoft power point. Additionally, the *ASPM* gene exons and intronic regions were also compared.

Evolutionary Rate Selection Pressure Test

Both gene and protein sequence alignments were subjected appropriately to various statistical tests in order to delve out the nature of selection pressure directing the adaptive evolutionary course of the gene. To explore the nature of selection pressure, the gene sequence alignment were subjected to Null hypothesis of all three selections, negative selection (HA:dN>dS), positive selection (HA:dN<dS) and neutrality (HA:dN=dS), where dN is the number of non synonymous mutation which dS is the number of synonymous mutations. The hypothesis was tested using Codon-Based Z-Test along with a mathematical model of Nei-Gojobori (p-distance).^{22,23}

RESULTS AND DISCUSSION

The Neighbour joining consensus tree based on protein sequences of *ASPM* gene, shown in Fig.1, is supported by high boot strap values indicating the identical branch and clades segregations during boot-straping stages. The tree clearly excluded all the out groups (non mammalian version of *ASPM* protein). Importantly, the tree holds the established evolutionary lineage of mammalian evolution, showing radiation of monotremes (*Ornithorhynchus*) before the rest of the advanced mammals. This is followed by the separation of rodentia (*Mus* and *Rattus*) clade. Further to it is the separation of taxa belonging to artiodactyla (*Bos*, *Hippopotamus* and *Ovis*) and cetacean (*Tursiops*) in a single clade. It is worth mentioning that although Hippopotamus is taxonomically the member of order artiodactyla but several paleontological, physiological and molecular studies have unravelled that they are indeed the closest relatives of cetaceans like Dolphins and Whales.^{24,25} Moreover, both artiodactyla and cetaceans shares a common ancestor (cetaartiodactyla) and split between these two order occurred around 60-55 million years ago (mya). Taken these facts into account, the clustering of Hippopotamus with Dolphin in the tree based on *ASPM* protein sequences provides yet another piece of evidence regarding the evolutionary relatedness of *Hippopotamus* and cetaceans. Following that is the branch leading to *Equus* (a member of Perisodactyla) which also radiated from the common ancestors of cetaartiodactyla, namely ungulatomorpha in late cretaceous or early paleocene period (70-64 mya) however, they only started diverging around 10-15 million years later than the earlier split.^{26,27} Similarly, the tree also agrees with the notion of relatively recent diversification of carnivores than perisodactyla. Indeed, the split between the common ancestors of cetaartiodactyla and ungulatomorpha and members of order carnivora (*Felis*, *Canis* and *Ailuropoda*)

occurred in the early paleocene period (80 mya) but their diversification had started around 55 mya.^{26,27} As anticipated, the primates formed the separate clades after all the non primate mammals, which is in agreement with the established time scale of separation of primates and other mammals from their common ancestors. Albeit the split between the common ancestors of primates (including human) and all the mentioned mammalian orders occurred during late cretaceous period or perhaps much earlier (>65mya) but the primates only diversified after Eocene period (<34mya) resulting the late separation of the primate clades than rest of earlier mammals. Akin to the well accepted notion of primate evolution, which is based on paleontological and molecular data (not ASPM), the ASPM phylogeny also validates the segregation of new world monkeys (*Callithrix*, *Aotus* and *Saimiri*) before the old world monkeys (*Colobus*, *Chorocebus* and *Macaca*). According to geological time scale, the split between the common ancestors of old and new world monkeys occurred at about 45 mya (Eocene period). The later split in the common ancestors of old world monkeys and rest of relatively advanced primates (*Hylobates*; Gibbon) happened at the late Oligocene period (around 25 mya). The further separation between *Pongo* (Orang utan) and Gibbon occurred in Miocene period (18 mya). *Gorilla* and other African apes including Human share their common ancestors with Orangutan around 14 mya. Common ancestor of both Chimpanzee (*Pan*) and Human (*Homo*) splits from *Gorilla* at around 7 mya. Finally the chimpanzees and humans have taken different evolutionary course from their common ancestors around 6 mya (very late Miocene period).^{26,27,28} The greater congruencies between the established evolutionary lineage of the mammals and the constructed phylogenetic tree not only strengthen the standpoint of evolution by natural selection but also verify the fidelity of tree and the algorithm on which it is based. Moreover, higher bootstrap values and clear out grouping of non mammals from the tree strongly implicates towards the validity of the phylogenetic calculation.

In human, *ASPM* gene is located on chromosome no 1, the largest of all human chromosome bearing 8% of our total genetic information.²⁹ Genetically, *ASPM* gene in all mammals are respectively flanked by Zn finger and BTB domain 41 (ZBTB41) and coagulation factor XIII genes (F13B) in 5' and 3' directions. However, the orientation of the gene changes during the course of evolutionary history. Both in Humans (*Homo*) and Chimpanzees (*Pan*) the orientation of all three genes remains same, however, it is found reversed in a representative of the old world monkeys (*Macaca*). The similarity of ASPM order between human and chimpanzee could be inferred in terms of the interbreeding era (roughly 3 million years) since the divergence from the common ancestor (6 mya)²⁹. As the old world monkeys and advanced apes have split from their common ancestor in the Oligocene period, thus the event

of this genes inversion must be not older than 25 million years from the present time. It is interesting to note here that the orientation of all three genes (*ASPM*, *ZBTB41* and *F13B*) are same in old world *Macaca* and wolves (*Canis*; Dog family) and mice (*Mus*). However, the orientation of *ZBTB41* and *ASPM* are found reversed in Cow (*Bos*). This implies that the inverted orientation in this lineage could have happened during the divergence of artiodactyla (<55mya) (if only found in cow and/or few members of the clade). Alternatively, it may be ancient duplication (>65mya) (Fig. 2).^{27,28,29} In addition to the gene order changes, subtle changes have also been found within the *ASPM* gene. Fig. 3 clearly represents the differences among the distribution, numbers and sizes of exons and introns in the *ASPM* gene among the compared mammals including human. Architecturally, the diversity of the eukaryotic genome is primarily mediated by the sequence divergence, insertion, deletion, duplication and recombination³⁰, the mentioned changes in the synteny and exon/intron pattern in *ASPM* genes could be the function of any/some or all of such processes. Additionally, expression of the gene is known to be affected directly or indirectly by gene synteny and/or orientation and pattern of splicing.^{31,32} Taken together, it is possible that the order and perhaps more importantly the intragenetic architecture may influence the *ASPM* protein expression positively in the humans resulting in the extraordinary encephalization and increment to the intellectuality and cognitive abilities. Supportive to this inference is the study conducted by Caceres and co-workers, which has shown the exceptional transcriptional edge in human as compared to other primates.³³ Additionally, the sequential changes in *ASPM* gene after the split from the common ancestors of chimps could not be discounted as it is known that human encephalization had occurred several times in its evolutionary history, starting around 2 mya and reaching its current status approximately 500,000 years ago.^{14,34}

Codon based Z test clearly shows the expected positive selection of the protein as one move from rodentia to primates. Importantly, most of the significant values lies in the clade of old world monkeys (clade where humans reside). The values imply that non synonymous mutations (change in amino acid) in the *ASPM* protein are tolerated and in certain cases even favoured in the nature. However, this case of positive selection could not be implemented to the mutations of *ASPM* gene causing microcephaly, as mutations responsible for microcephaly are protein truncation mutations thus producing an incomplete protein.³⁵ Our finding is in line with some earlier investigations regarding *ASPM* evolutionary pressure^{6,14,16}, however, the studies have mainly confined to the selection pressure present in only primate lineage. Maximum Composite Likelihood Estimate has shown that the transition over transversion, however, with few exceptions this trend considered to be universal among metazoans.³⁶

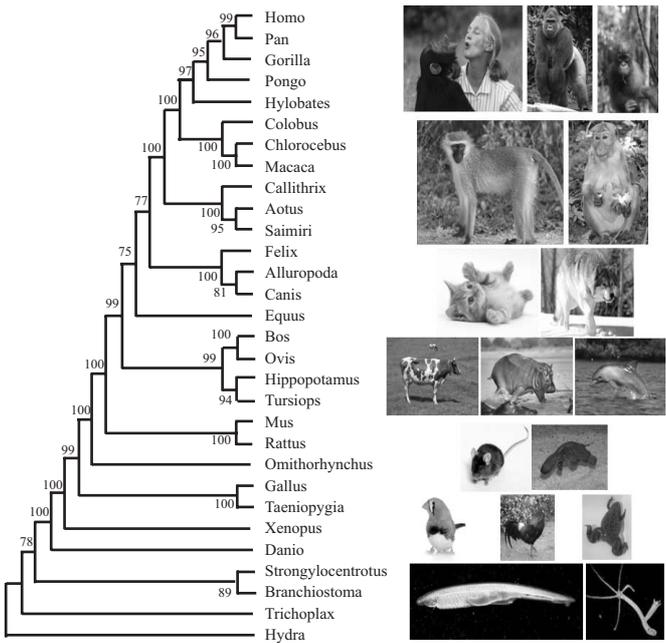


Fig. 1. Evolution of ASPM protein: Phylogenetic tree constructed on the basis of ASPM protein sequence alignment using neighbour joining method. Clades of different taxa are differentially coloured. Orange branches are of out groups (non mammalian ASPM), brown branch represents monotremes, Purple clade include members of cetartiodactyla, blue for perisodactyla, orange for members of carnivore and green clade constituted on the members of primates including human. Boot strap values are shown at each branch point. (For details please see text).

CONCLUSION

Taken together, we believe that substantially high intelligence in the primates, especially in humans, is the result of gradual modifications within and outside the brain development genes like *ASPM*. Such modifications are rigorously scrutinized by the forces of natural selection and favoured if found benevolent for the survival of the organism individually and in many cases for the entire species. We realize that the empirical data in connection to *ASPM* for such notion is lacking. Additionally, we are broadly unaware of the structure-function aspects of ASPM protein, once known, it may clarify the significance of minute sequential changes among the ASPM protein of different mammals in terms of their structure and consequently their functions. However, intellectual superiority of humans over other living species could not be fully answered by single gene or set of genes. Evolutionary Medicine is a new emerging field of biology, it is plausible to expect that soon in combination with the system biology and transgenic animal models we would be able to answer this great mystery of nature.

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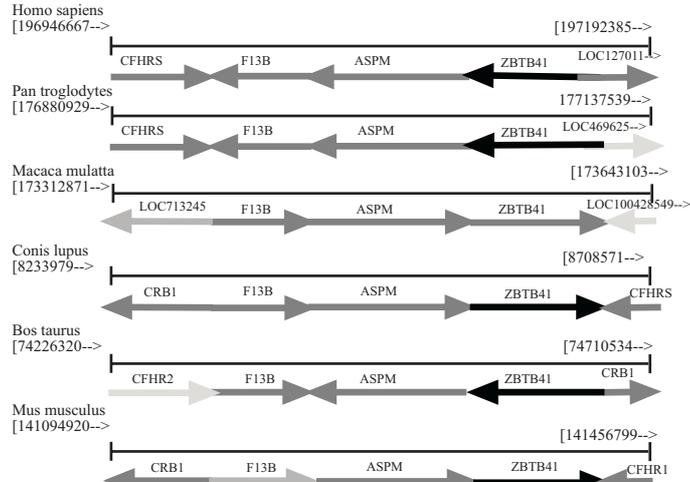


Fig. 2. Gene synteny of ASPM: Gene orientation of ASPM (Red arrow) and its flanking genes Zn finger and BTB domain 41 (ZBTB41; black arrow) and coagulation factor XIII genes (F13B; green arrow) is shown, head of arrows indicates the direction of transcription. Note the change in the orientations among different mammals which may affect the rate of expression of the ASPM, details are furnished in the text.

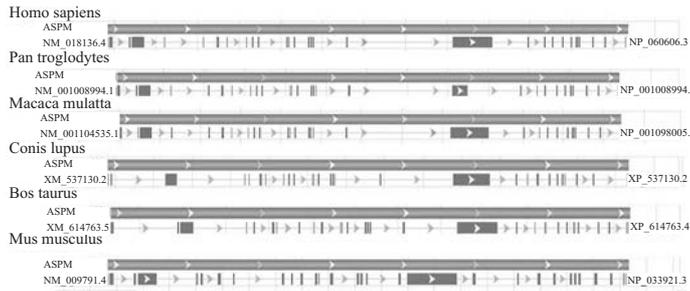


Fig. 3. Distribution of exons and introns of ASPM gene: The pattern of exons and introns in the ASPM varies considerably in terms of size, number and location within the gene among different mammals. Green bars, rectangles and squares indicated underneath the gene are the exons while the lines in between them are introns.

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Two Cases of Ambiguous Genitalia

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ABSTRACT

The term “Ambiguous genitalia” applies to confusing appearance of the external genitalia. Sex assignment becomes essential for the parent’s peace of mind, and in turn depends on anatomy and functional endocrinology rather than karyotype.¹ Two cases with all different genetic sex, gonadal sex and phenotypic sex are described.

First case is that of congenital adrenal hyperplasia (CAH) in a month old baby whose genotype was female with laboratory investigations exposing her diagnosis. She is doing well with oral hydrocortisone and fludrocortisone. Second case is that of probable 5-alpha reductase deficiency who would probably need future surgery.

Key words: Ambiguous genitalia, congenital adrenal hyperplasia (CAH), 5-alpha reductase deficiency, intersex.

INTRODUCTION

Intersex conditions vary in frequency. CAH is the most common cause of ambiguous genitalia in the newborn. Mixed gonadal dysgenesis (MGD) is the second most common cause of intersex conditions. Clinicians should suspect the possibility of an intersex condition if hypospadias and cryptorchidism occur in the same patient as 50% turn out to be intersex conditions.²

Disorder of Sex Development (DSD) is the new terminology replacing intersex with their main advocate being the intersex society of North America in order to avoid conflating anatomy with identity.³ This has been accepted in a consensus meeting on management of intersex disorder.^{4,5,8,10}

Genital ambiguity includes an infant with:¹

1. A phallus but bilaterally unpalpable testes.
2. Unilateral cryptorchidism and hypospadias
3. Penoscrotal or perineoscrotal hypospadias even if the testes are descended.

Male phallus needs to be 2.5cm in length and clitoris <1cm with no posterior fusion in order to be labeled normal.¹

Early intervention has been criticized by experts citing individual’s experiences of interventions and the lack of follow-up studies shows clear benefits.⁶⁻¹⁵

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Modern treatment of infants with ambiguous genitalia involves a multidisciplinary approach. This gender-assignment team usually involves neonatologists, geneticists, endocrinologists, surgeons, counselors, and ethicists. The goal is to provide appropriate medical support and counseling regarding care and therapy. The topic of early gender reassignment is currently under debate.²

CASE NO. 1

Sabeen a 30-days-old baby was referred from a private hospital with complaints of undetermined sex. Mother was a booked case with regular antenatal visits. She had routine ultrasound (obstetrical) examinations, she was prescribed oral iron and folic acid supplements during pregnancy. Beside, she also received regular antiepileptics (Phenobarbitone and Epival) for epilepsy treatment. Delivery was uneventful as was the post natal period. She is the 4th product out of a consanguineous marriage. On examination the child was active, alert with stable vitals (H/R: 120 beats/min, R/R: 40 breaths/min, Afebrile, BP: 55/35mmHg) and anthropometrically WNL (Ht: 54 cm, Wt: 2.5 kg, FOC: 35 cm). Examination of genitalia revealed phallus size: 1.3 cm, labioscrotal folds fused, gonads not palpable, single opening at the base of phallus. Systemic examination was unremarkable.

Working diagnosis was that of a virilized female. Investigations revealed 46xx karyotype, Ultrasound pelvis revealed uterus present measuring 3.2 x 1 x 1.8 cm, right ovary measuring 1 x 0.75 cm, left ovary 1 x 0.8 cm ie normal uterus & ovaries. Serum electrolytes were Na⁺ 139meq/l, K⁺ 5meq/l., 17-OH Progesterone: 33.7 ng/ml(n= 0.20- 3.30), Serum Renin: 34.59 ng/ml/hr(n= 0.15 – 2.33)

Diagnosis of CAH was made and immediately hydrocortisone and fludrocortisone was started. Her labs improved with the treatment. A good follow up plan was communicated at discharge.

CASE No. 2

3-years-old Humaira weighing 13kg came with fever for 15 days. Ambiguous genitalia noticed by mother at age of 2 months. On examination she was vitally stable with good anthropometric measurement. Local examination revealed bilateral partially fused labial swellings, stretched phallus size 1.5cm, single ventral urethral opening, gonads are bilaterally palpable in labial swellings either is around 1.5 cm, no rugae pigmentation at labia, no vaginal opening with anus normal.

Investigations showed uterus, ovaries and vagina were not visualized, hypoechoic areas in lower part of both inguinal regions likely testicular tissue, maximum diameter was 1.5 cm on either side on ultrasound pelvis. MCUG showed well developed elongated male type urethra and no Mullerian remnants. Karyotype was 46xy, Base line testosterone was < 20 ng /dl (male 0-4yr 10 -160 and female <12yr upto 20 normally). After HCG stimulation S. testosterone 44.9ng/dl. Final diagnosis was made of an Undervirilized Male with 5-alpha reductase deficiency or Partial Androgen Insensitivity Syndrome

Management plan advised multidisciplinary consultation with endocrinologist, pediatric surgeon & urologist with a view to reconstructing the child for male sex.

DISCUSSION

At around eight weeks of gestation, under the influence of gene located on Y chromosome (SRY gene) the gonads of an XY embryo differentiates into functional testes, secreting testosterone. Ovarian differentiation, for XX embryo, does not occur until approximately week 12th of gestation. In normal female differentiation, the Mullerian duct system develops into uterus, fallopian tubes and inner third of the vagina. In males the Mullerian duct inhibiting hormone (MIH) causes this duct system to regress. Next, androgen causes the development of the Wolffian duct system, which develops into the vas deferens, seminal vesicles, and ejaculatory ducts.¹⁶

Causes of ambiguous genitalia are virilization of female infant and undervirilization of male infant.

Causes of undervirilization of male infant are, defect in testicular differentiation, testicular hormone and androgen action.

Defects in testicular differentiation include, Deny –Drash syndrome, WAGR syndrome, Camptomelic syndrome, XY pure gonadal dysgenesis and XY gonadal agenesis.

Defects in testicular hormone includes, Leydig cell Aplasia, Congenital adrenal hyperplasia.

Defects in androgen action may either be, 5 alpha reductase deficiency or Androgen insensitivity syndrome.

Congenital Adrenal Hyperplasia is a rare autosomal recessive disorder. Cortisol deficiency increases ACTH causing adrenal hyperplasia and overproduction of intermediate metabolites. Depending upon the enzyme deficient, there may be clinical and laboratory findings of mineralocorticoid deficiency or excess, incomplete virilization, or premature puberty in affected males and virilization in affected females.

More than 98% CAH causes are due to 21 OHLase deficiency. Classic type occurs in 1 in 15000- 20000 births. 75% of infants have salt losing form. 25% have simple virilizing form. Non- classic has prevalence of 1/1000 in general population (more common in Jews) shows mild elevation of androgens & signs of androgen excess postnatally. Decreased production of cortisol leads to loss of feedback on pituitary causing increased ACTH leading to stimulation of adrenal hyperplasia giving increased production of steroids above blockage including androgens.

Excessive androgen production leads to virilizing symptoms in females. Progressive weight loss, anorexia, vomiting, dehydration, weakness, hypotension, hypoglycemia, old hyponatremia, hyperkalemia. Prenatal androgen excess causes affected females to have masculinized external genitalia, enlargement of clitoris, labial fusion, and common vaginal & urethral opening (urogenital sinus). Severity of virilization is greatest in females with salt losing form of 21 OHLase deficiency. Internal genital organs are normal.

Prenatal exposure of brain to high levels of androgens may lead to sexually dimorphic behavior in affected females. Males appear normal at birth. Diagnosis can not be made until signs and symptoms of adrenal insufficiency develop. Postnatal androgen excess causes rapid somatic growth, accelerated skeletal maturation. Affected patients are tall in childhood, but premature closure of epiphysis causes growth to stop early, adult stature is stunted. Muscular development is excessive. Pubic & axillary hair may appear. Acne & deep voice may develop. Patients with non classic type have normal genitalia at birth, but may present with precocious puberty.

Investigations for CAH include Karyotyping, U/S pelvis, Serum electrolytes, 17 OH Progesterone, Renin, Aldosterone, ACTH.

Prenatal diagnosis in parents already having affected child can have CVS late in 1st trimester for analysis of DNA; during 2nd trimester by amniocentesis. The goal is to facilitate appropriate prenatal treatment of affected females by Dexamethasone 20ug/kg daily (2-3 divided doses) which suppresses secretion of steroids by fetal adrenals.

Treatment of case is hormone replacement by Oral Hydrocortisone 10-20 mg/m²/24hrs in 3 divided doses; double or triple doses indicated during periods of stress such as infection or surgery. Monitoring growth along percentile lines, higher height percentile indicates under treatment, loss of height percentile means over treatment. Excessive weight gain suggest over treatment, periodic pubertal development monitoring, serial x-rays of hand & wrist for bone age. Fludrocortisone : 0.1-0.3mg (2 divided

doses) with sodium supplements. In older children 0.05-0.1 mg daily is adequate. Monitoring serum electrolytes frequently especially in early infancy, and plasma renin level. Surgical management of ambiguous genitalia is done usually at 4 -12 months of age.

Case 1 was that of typical CAH 21OHLase deficiency type with virilized female proven by karyotyping, imaging and biochemistry. She was advised lifelong salt supplements along with oral hydrocortisone and fludrocortisones and a life long monitoring plan.

5 alpha reductase deficiency as an autosomal recessive disorder. In neonate phenotypic findings are limited to genitalia. External genitalia exhibit labial appearance to labioscrotal folds with mild roagation or pigmentation, phallus size fall between 1 to 2 cm, urethra may open from tip of phallus to perineum, uterus fallopian tubes and vagina are absent.

In Androgen Insensitivity Syndrome (AIS) the features of partial insensitivity vary from infertility to hypospadiasis, hypogonadism unilateral or bilateral cryptorchidism. In complete AIS, internal genitalia are of male type external are female type, frequently bilateral inguinal hernia are present, testis may be any where along their pathway. Risk of gonadoblastoma is about 20%.

Workup shows XY karyotype, low LH and low Testosterone / Dihydrotestosterone (DHT) in hypothalamopituitary defect, normal to high LH with normal testosterone but low DHT in 5 alpha reductase deficiency and in androgen insensitivity high LH low T / DHT is found. In healthy pre pubertal children baseline T/DHT ratio is 1:2 after HCG stimulation it become 8:1 to 14:1. Imaging studies include ultrasonography, genitogram / vaginogram, also gonadal biopsy is sometimes indicated.

Gender assignment should be deferred until the initial diagnostic evaluation has been completed and the condition clarified. Treatment includes hormone replacement therapy, psychosocial support and reconstructive surgery.

The 2nd case was that of 5-alpha reductase deficiency supported by karyotyping, imaging and relatively normal testosterone levels. DHT levels are not done here so final diagnosis cannot be confirmed nor can partial androgen insensitivity be ruled out. Only time can tell the ultimate fate of such cases as hormonal potentials become evident during puberty time.

In short management of ambiguous genitalia needs timely and multidisciplinary approach and a life long followup plan.

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CASE REPORT

Spontaneous Per Urethral Expulsion of Gossipibyoma – A Rarity

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and Muhammad Shahab Athar¹

ABSTRACT

Foreign bodies left accidentally during a surgical procedure are rare. In spite of careful intra-operative precautions and gauze counts mistake still occurs. Many of these cases present with sepsis, the foreign body may erode hollow viscera with and without signs of peritonitis and discovered on laparotomy. These foreign bodies may remain silent for years. We are reporting an interesting case of an 11 years old girl who presents with gauze piece coming out of her urethra 4 years after cystolithotomy and right ureterolithotomy. Such cases of intraluminal migration and spontaneous expulsion of foreign body are rarely reported.

Key words: Foreign bodies, Gossipibyoma, Retained foreign bodies, Retained surgical sponge.

INTRODUCTION

Foreign bodies left accidentally during a surgical procedure are rare. These foreign bodies are of varied types and range from instrument to gauze piece. Among them surgical sponge is the most common. They account for less than 0.01 % of abdominal surgeries¹. Such incidents are more commonly seen in general surgical, gynecological, obstetrical and urological surgeries but can occur in any surgical specialty².

These foreign bodies usually induce foreign body reaction and present early, but they may remain silent for years and present as pseudotumor called gossipibioma. Many times these foreign bodies erode nearby hollow viscera and present with signs & symptoms of peritonitis or sepsis and the foreign body are discovered on exploration³. These left over foreign bodies often have severe medicolegal issues.

Herein we are reporting rare case of a girl who had cystolithotomy & right ureterolithotomy, she presented 4 years later with ribbon gauze protruding from her urethra.

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CASE REPORT

An 11 year old girl presents in a private hospital with the complaints of something coming out of vulva for 2 hours. She also had severe dysuria, increased frequency of micturition and urinary incontinence for the last 4 days. She had past history of cystolithotomy and right ureterolithotomy 4 years back in the same hospital by someone else. She had an attack of urinary tract infection 2 months back that settled down on oral medications.

For the last 4 days she had severe dysuria, increased frequency of micturition and episodes of urinary incontinence. For this she went to a urologist who further investigated her. At that time her urinalysis showed numerous pus cells and red cells. X-Ray KUB revealed a radiopaque calculus in the bladder (Photograph - I). Ultrasound KUB showed thick walled urinary bladder with multiple calculi. On the bases of these she was diagnosed as a case of recurrent vesicle calculus. She was catheterized and sent home with the plan of cystolithotomy after 5 days as the consultant was going outstation.

In the morning she took out her catheter with the help of a nurse on her own, as she was irritated with it. After few hours she noticed something coming out of her vulva. She tried to pull it out but it was painful, so she was brought to the hospital by her parents.

On examination a piece of gauze 3 to 4 cm long was protruding from the urethra. As the child was irritable it was planned to remove under general anesthesia. On examination under anesthesia the gauze piece was found coming from the external urethral meatus, the hymen was intact (Photograph - II). When it was pulled out a piece of ribbon gauze about 10 cm long came out and on its distal end a calculus 2 x 1 cm in size was attached that also came out along with it (Photograph - III).



Photograph 1: X-Ray Pelvis Showing Radiopaque Calculus in the Urinary Bladder



Photograph 2: Ribbon Gauze Coming from the External Urethral Meatus, and Intact Hymen



Photograph 3: Ribbon Gauze Piece Removed From The Urinary Bladder Along With Calculus Attached At Its Tip

Post operative X-Ray KUB was normal, however her ultrasound KUB still showed some foreign body in the urinary bladder. Her cystoscopy was then performed and a second piece of ribbon gauze about 4 cm long was again removed. The anterior wall of the bladder showed a small hyperemic area. Later her C.T. scan of pelvis was performed and was found to be normal.

DISCUSSION

Foreign bodies left during surgery have serious implications. Although it is rare, it may vary from sponge to instrument. Surgical sponge is the most common foreign body left over during surgery. It has been reported to occur following abdominal, thoracic, gynecological, orthopedic, and even neurosurgical operations, but can occur in any field of surgery². It is more common in abdominal and gynecological surgeries where deep cavities are explored especially in obese patients and when the surgery was performed in emergency situations⁴.

Surgical sponge or gauze piece left during surgery can be potentially life threatening and has serious medico legal implications. Although it is rare, the exact incidence is not known. It is estimated to account for less than 0.01 % of intra abdominal surgeries¹.

These retained gauze piece induces a foreign body reaction in the in the surrounding tissues and depending upon whether they are sterile or infected presents early or late. They produce diagnostic difficulties as their presentation is quite varied³. They may present early in immediate postoperative period as continuing sepsis or they may remain silent for years. Later granuloma may form around them and presents as abdominal mass mimicking a tumor (Pseudotumor) this tumor like mass is called Gossipyoma. Clinically it is very difficult to accurately diagnose them⁵.

Sometimes these left over foreign bodies erodes a neighboring hollow viscus and presents as sepsis or fistula formation even after years of operation such sepsis may be life threatening^{6,7}. In addition intraluminal migration and spontaneous expulsion of such foreign bodies are rarely reported.

In our case after cystolithotomy and right ureterolithotomy the patient remain asymptomatic for 4 years. Her symptoms started 2 months back as increased urinary frequency and dysuria which was diagnosed and treated as urinary tract infection. Later she develops the same symptoms that progress to urinary incontinence. At that time she was seen by a urologist who investigated her and the reports at that time revealed stone in the bladder. The X- Ray picked the secondary stone formed on the foreign body; it was unable to pick the gauze piece as it was not labeled with radio opaque marker. Similarly the sonologist was unable to pick up the foreign body initially probably because it was overshadowed by the echoes produced by the secondary calculus. Later after removal of the prolapsed gauze piece along with the stone the sonologist was able to detect residual gauze which was removed cystoscopically. The hyperemic area in the anterior wall of bladder is probably the site from where the sponge has entered.

Although extrusion of retained surgical sponge in to a hollow viscus (Transmural migration) is known^{6,7} but intraluminal migration is a rare phenomenon. Cases were reported where laparotomy sponge extrude into the bowel lumen without clinical signs^{8,9} and migrate distally causing intestinal obstruction^{8,9,10}. Similarly migration of surgical sponge into the urinary bladder after herniorrhaphy had also been described^{11,12} but spontaneous extrusion through the bladder is rare and as far as our knowledge is concerned and after thorough literature search no such case has been found.

The retained foreign bodies represents serious ethical and legal dilemma. Although it is serious, but can be avoided. The whole surgical team has to be vigilant especially during closure. This problem can be prevented by: 1- Careful handling of gauzes during surgical procedures. 2- Thorough double counting of the gauzes before closure of wound and at the end of operation. 3- Thorough exploration of the surgical site at the end of procedures 4- Routine use of sponges and gauzes impregnated with radio opaque marker. 5- Routine radiographic screening of high risk patients before they leave the operating room, even when the gauze counts are documented as correct¹³.

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A Simple Method Adopted for Tooth Sectioning in Dr. Ishrat-ul-Ebad Khan Institute of Oral Health Sciences, DUHS

Amynah Charania¹, Humaira Akhlaq² and Zia Iqbal³

INTRODUCTION

From the third world countries including Pakistan, need for effective techniques for the study of histological tooth sectioning at undergraduate level is lacking. This is primarily due to poor resources.

Identification of human remains has always been a challenge for the forensic personnel,^{1,2} however identification through tooth sectioning may be a useful tool. The importance of tooth sectioning is realized in disasters such as earthquake, floods, airplane crashes, cyclones, volcanic explosions and terror acts etc. Also age estimation in living individuals with no valid proof of date of birth is on rise. Many medicolegal solutions are possible just by studying teeth.³⁻⁸ Histological details are important to study the morphology of oral tissues as well as for the identification of pathological conditions.^{9,10} Thin sections of teeth also provide an excellent tool for dental research.

There are many methods to look for anatomy of teeth and depending upon them various types of sectioning is adopted. Routine methods to study the morphology of oral tissues involve preparation of tissue, sectioning, staining and then observing under microscope, a procedure known as Tissue Processing. It can be defined as any treatment of hard or soft tissue necessary to keep it in a life-like manner and to impregnate it with a solid medium so as to give enough rigidity to facilitate the preparation of thin sections for microscopic study.

Methods commonly used for oral tissue preparation are:

- a) Preparation of sections of paraffin-embedded specimens
- b) Preparation of sections of parlodion-embedded specimen

- c) Preparation of ground sections of teeth
- d) Preparation of frozen sections

Procedure for tissue processing involves fixation of tissue, dehydrating in organic solvent, embedding in paraffin wax and then cutting on a microtome. Sections are then mounted on a slide, stained with haematoxylin and then observed under microscope.¹¹ The basic procedures are modified depending on the nature of specimen and the type of microscope to be used for examination.⁹ Many types of microscopes are used for the study of oral tissues, but most commonly used one is the bright field light microscope with a compound lens system.¹¹

As mentioned earlier, we in our setup lack proper resources, for elaborate and expensive lab equipments. Thus simple and alternate procedures may be adopted so that effective teaching at the undergraduate level may successfully be achieved. A simplified cost effective technique is given which was used before but usually not carried out nowadays as newer gadgets (expensive) have replaced the older simpler methods. We being a part of third world nation may adopt the simplified method due to our limited resources.³

In addition, some authors suggested that decalcification-sectioning procedure is often too harsh for archaeological remains and other researchers have found that decalcification tends to produce macerated sections in archaeological materials.¹²

Thus for study purpose at undergraduate level we can prepare histological ground sections of tooth by the help of model trimmer (carborundum stone) instead of using expensive microtome because it is easily accessible, less expensive, simple and one of the oldest method.¹³ The greater advantage of this method is the possibility to distinguish between mineralized and unmineralized tissue by an excellent preservation of tissue. Tooth enamel has 96% of minerals, which are destroyed by ordinary methods of decalcification. Thus, at undergraduate level, microscopic study of ground section of tooth by model trimmer may be one of the satisfactory methods for the preservation of calcified tooth structure. Un decalcified teeth may be studied by making thin ground sections without using any chemical in contrast to routine microtome preparations which involves decalcification, where most of the enamel is lost and histological details are often obscured.¹⁴⁻¹⁶

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Undecalcified ground sections of tooth is also useful for simultaneous examination of mineralized dental tissue covering the root surface of teeth with periodontal disease.¹⁷

MATERIALS & METHODS

100 teeth extracted due to orthodontic or periodontal reasons were collected from the surgery department of Dr Ishrat ul Ebad Khan Institute of Oral Health Sciences for preparation of ground sections. Teeth included were anteriors and posteriors consisting of 26 incisors, 9 canines, 45 premolars and 25 molars. All grossly decayed teeth were excluded from the study. Other materials required included hard plaster, rubber bowl, plaster spatula, and plaster cutter, glass slab, model trimmer (carborundum stone), Arkansas stone, bench grinder, pumice powder, glass slide, D.P.X solution, cover slip and microscope.

Each extracted tooth was cleaned with hydrogen peroxide to remove the debris on the tooth surface and then washed with running water thoroughly.

Making A Plaster Slab:

A plaster slab of approximately 4x3" was made and the cleaned tooth was embedded (mesiodistally & labiolingually) in the center of the slab in such a way that half of the tooth was embedded while half of it was outside.



Embedded tooth in plaster slab

Preparation Of Ground Sections

To make a ground section, the tooth was trimmed longitudinally in a labiolingual / mesiodistal plane with the help of model trimmer. Plaster slab was placed on the grinding surface of the trimmer and held with one hand from the back of the slab with medium to light pressure. Constant cooling was observed by water spray during grinding to avoid generation of excessive heat and minimize the damage. The tooth was ground down to the desired width (approx 4-5 mm) on both sides. This section was removed from plaster slab and replaced by bench grinder with slow speed further till 2-3 mm. and then to about 1mm. Thus the coarse abrasive wheel was replaced by fine abrasive wheel and the ground surface of the cut tooth is held firmly against the rotating wheel. The tooth was soaked in water throughout to avoid dehydration and cracking of the section.



Model trimmer for initial preparation



Bench grinder for final finishing

Final Finishing and Polishing

Final polishing was done to remove irregularities on tooth section. Slurry of pumice and water was then rubbed with the section on Arkansas stone to make it smooth and paper-thin (approx 0.25 mm).



Arkansas stone for final finishing and polishing

Slide Preparation

A clean slide and cover slip was taken and the section was placed in the center of the slide. The cover slip was mounted by using a drop of D.P.X. solution. The slide was ready for examination under the microscope.



(Prepared ground section slide of a canine tooth)



(Prepared ground section slides of anterior and posterior teeth)

RESULTS

Observation of Slide

The prepared thin ground section was examined for different calcified tooth tissues namely enamel, dentine and cementum under microscope.



(Examination of prepared ground section slide under microscope)

CONCLUSION

For undergraduate students this is one of the effective methods for preparation of ground sections in which the hard tissue details are preserved. The setup used was not very elaborative but was easily available with low cost. The normal anatomy and constituents of tooth was maintained by this method. On the other hand sections prepared by microtome would be expensive, time consuming as the procedure involves decalcification and staining quality of tissue is reduced due to prolonged exposure of acid. Preservation of cellular structure may not be achieved successfully.

Advantages of Ground Sections

This method is useful for the examination of mineralized tissue.

It is a simple method.

No expensive equipment is required.

Can be easily performed in the laboratory.

Decalcification of tooth is not needed.

Disadvantages of Ground Section

Hand grinding is injurious, but it is not replaceable with any other simple methods thus care should be taken during preparation.

Cracks or distortion of the sections may occur during grinding.

Bubbles under cover slip may form when the mounting medium is too thin.

All the soft tissues are lost during grinding.

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