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ORIGINAL ARTICLE

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Prevalence and Antibiotic Susceptibility Pattern of Staphylococcus Aureus in a Tertiary Care Hospital of Islamabad Pakistan

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Abstract

Background: Staphylococcus aureus mainly inhabits the skin and the mucosa of humans. It is regarded as a potential pathogen with the capability of causing diversity of infections after gaining entry to the host cell, initial mild infections of the skin can lead to invasive infections which can be threatening to life. Unlike other types of staphylococcus aureus, MRSA is challenging to treat because of its multidrug resistivity, the phenomena of antibiotic resistant in bacteria is called as "super bug".

Objectives: This study is planned to find out the anti-microbial susceptibility patterns and prevalence of staphylococcal infections in wound, pus, urine, and catheter tips samples in HBS hospital. Another aspect of our study is to find out the male and female ratio prevalence of SA in males and females & the male and female ratio of MRSA,

Methodology: This descriptive and prospective study was conducted at dept. of Pathology, HBS Medical &Dental College Laboratory from December 2017 to April 2019.

Overall samples received in the lab were 150 in number; in the microbiology section Swabs from wound, umbilical cord, eye, ear, throat, skin, and wound were considered for further analysis. Similarly, isolates of staphylococcus aureus from blood culture were collected and further analyzed. Isolates from a urine specimen, pleural aspirate, urine, high vaginal swab was also collected. S. aureus was isolated from samples of both male and females, belonging to all age groups of out or in patients hospitalized in different wards. These isolates were grown on MacConkey and blood agar media incubated for 24 hours at 370C. The susceptibility of all isolates was determined.

Results: Out of total 108 isolates of *S.aureus* 48(44.4%) were obtained from male and 60(55.5%) were from females. About 40.7% isolates resistant to cefoxatin and oxacillin were methicillin resistant *S.aureus* (MRSA) whereas 44.4% isolates sensitive to cefoxatin and oxacillin were methicillin sensitive *S.aureus* (MSSA).

Conclusion: Resistance to antibiotics has undoubtedly risen to extremely high levels. Certainly, the antibiotics should be prescribed cautiously for the treatment based on results of culture reports infections. This would help elude the consequences of the increase of Resistant *S. aureus* in our settings. Good hospital infection control measures prove to be the main stay against these infections because antibiotics can never be an effective alternate to good medical practice.

Keywords: *S.aureus*, MRSA, Resistance pattern

Conflict of Interest: None **Funding Source**: None

Introduction

Staphylococcus aureus causes various infections. Staphylococcus aureus is gram positive coccus, it is known to be causing agent of infections of skin and soft tissue such as furuncle, carbuncles, boils, and skin abscesses. The infection caused by *S.aureus* may start from a small boil forming abscess extending to bone causing osteomyelitis, it can also cause disseminated infections of heart valves such as endocarditis. It can also cause urinary tract infections and bacteremia.[1] Positive catalase and coagulase reactions are shown by *S.aureus*.[2] As the organism is found as normal flora of the skin, nose, and nasopharynx it is likely to be spread through air and it can likely also be transmitted by fomites.[3] It is assessed that 20% of the population are nasal carriers of this bacterium which makes it an effective pathogen, it also possess immune-evasive properties. Among the

species of genus staphylococcus, the most infective is S. aureus. It can cause serious diseases such as toxic shock syndrome (TSS) which is caused by a superantigen produced by the organism likewise pneumonia and endocarditis are also diseases caused by it.[4] *S.aureus* often causes post-surgical wound infections being susceptible to almost every antibiotic which are in use in medical practice.[5]

Antibiotic resistance strains of *S.aureus* have reached to a level that now it is thought that infection caused by such strains have taken a form of an epidemic around the globe. It is now well-known that resistant strains of S. aureus are continuously developing and are found commonly in hospitals & community settings. Mechanisms producing resistance comprise inactivation of antibiotics due to action of enzymes, reduced affinity for the antibiotics because of variations of the target, action of efflux pumps also by mechanism of antibiotic trapping. Higher rates of disease and mortality are associated with Multidrug Resistant Staphylococcus aureus (MRSA) strains.[6] This might be due to use of antibiotics without any restriction in a particular setting. This makes infections caused by these pathogenic bacteria found to be very difficult to manage and expensive to treat. Multi drug resistance 'Superbug", status of MRSA has been recognized because of the resistance shown by the organism towards vancomycin and other antibiotics which are not structurally similar, thus making it more harmful than ever in hospital settings and recently, in the healthy community.[7] The objectives of this study were to detect the prevalence and susceptibility of staphylococcal infection in tertiary care hospital and to identify the Methicillin/Oxacillin resistant S. aureus (MRSA, ORSA) from clinical specimens using common used antibiotics.

Methodology

After approval from ethical review committee of institute, this study was descriptive and prospective and was conducted at HBS Medical College (Microbiology department). A total of one hundred and fifty clinical specimens, isolates of Staphylococcus aureus were obtained. In the microbiology section Swabs from wound, umbilical cord, eye, ear, throat, skin, and abscess were further investigated using standard protocls. Similarly, isolates of staphylococcus aureus from blood culture were collected. Isolates from urine specimen, pleural aspirate, urine, high vaginal swab were collected between December 2017 and April 2019. Isolates of S. aureus were obtained from both genders and all age groups of out or in patients hospitalized in different wards.

The standard collection technique was used for Specimen's collection. The blood cultures were done using an (BACTEC 9240 and 9050 BD) which is an automated blood culture system afterwards bacterial growth examination was done. Specimens were inoculated on bacteriological media, Mac-Conkey and Blood agar Media which is an enriched media were used. After inoculation, the plates were incubated aerobically for 24–48 hours at 37 °C.

The isolates were identified by using standard methods. Primarily identification was done using basic methods: study of colony morphology, performance of Gram staining, biochemical tests like catalase and coagulase tests were done for further identification. Antimicrobial susceptibility testing according to CLSI guidelines, Kirby–Bauer disk diffusion method was used.

The antibiotic susceptibility pattern of all isolates was done against the following antibiotics: penicillin, Amoxicillin, Ceftriaxone, Cefixime, Cefizox, Cefoperazone, Cephradine, azithromycin, Imipenem, Meropenem, Ciprofloxacin, Ofloxacin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole, oxacillin, Methicillin, erythromycin, cefoxitin, linezolid, levofloxacin, tetracycline, clindamycin, and vancomycin. Antibiotic susceptibility tests were carried out by disc diffusion method. Using laboratory standards issued by national committee for clinical (NCCLS) The measurement of Zones of inhibition were made and compared. Resistant to methicillin isolates measured (<17mm) were named Methicillin resistant Staphylococcus aureus (MRSA), zone of inhibition as (17mm) were named susceptible.

Statistical Analysis: Data analysis was done with Statistical Package for Social Sciences (SPSS), version 21.0 (SPSS, Chicago, IL, USA) The differences in data at $p<0.05$ were termed statistically significant. Chi (X2) tool was used.

Results

By the use of different tests like culture, Gram staining and biochemical tests (coagulase and catalase), 108 clinical specimen of *S.aureus* were tested from different body sites. Among these clinical specimens 57(52.8%) from pus, 33(30.6%) from urine and 12(11.1%) were from blood. Other specimens include high vaginal swab 3(2.8%) and body fluid 3(2.8%).

Out of total 108 isolates of *S.aureus* 48(44.4%) were obtained from male and 60(55.5%) were from females.

About 40.7% isolates resistant to cefoxatin and oxacillin were methicillin resistant *S.aureus* (MRSA) whereas 44.4% isolates sensitive to cefoxatin and oxacillin were methicillin sensitive *S.aureus* (MSSA). The sensitivity and resistance pattern of different antibiotics was shown in table I. It was noted that most of MRSA were isolated from pus sample and then by urine samples. High susceptibility was seen with imipenem (92.5%).

High resistance was observed with penicillin (53.7%) and chloramphenicol (51.8%) and trimethoprim/sulfamethoxazole (53.9%). Imipenem showed high susceptibility pattern on isolates taken from all samples (Table III).

Table I: Frequency of s. Aureus according to sex.		
Organism	Male	Female
S.aureus	48(44.5%)	60(55.5%)

Table II: Frequency of *S.Aureus* In Clinical Isolates	
Specimen	Total S.aureus isolated
Blood Cultures	12 (11.1 %)
Pus	57(52.8 %)
Urine	33(30.6 %)
High Vaginal Swab (HVS)	3(2.8 %)
body fluid	3(2.8 %)

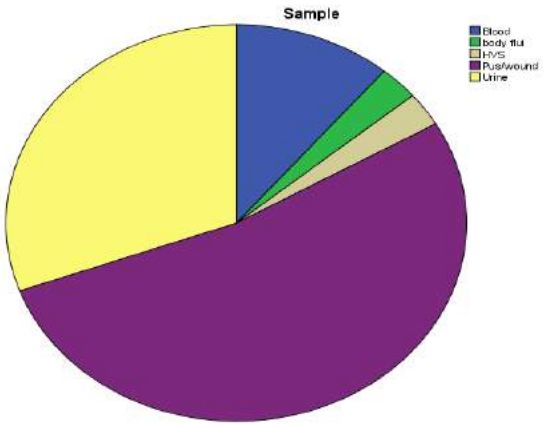

Figure 1. Distribution Pattern of Staphylococcus Aureus in Different Specimens

Table III: Antibiotic Susceptibility Patterns of *S.Aureus*			
Name of antibiotics	**Sensitive**	**Intermediate**	**Resistant**
Penicillin	42(38.8%)	08(7.4%)	58(53.7%)
Oxacillin	36(33.3%)	16(14.8%)	56(51.8%)
Imipenem	100(92.5%)	08(7.4%)	0
Cefoxitin	52(48.1%)	20(18.5%)	36(33.3%)
Clindamycin	52(48.1%)	20(18.5%)	36(33.3%)
Gentamycin	68(62.9%)	08(7.4%)	32(29.6%)
Tetracyclin	88(81.4%)	04(3.7%)	16(14.8%)
Chloremphenicol	48(44.4%)	04(3.7%)	56(51.8%)
Vancomycin	68(62.9%)	00	40(37%)
Co trimaxazole	40(37%)	O4(3.7%)	64(59.2%)
Ciprofloxacin	68(62.9%)	20(18.5%)	20(18.5%)
Linzolid	84(77.7%)	04(3.7%)	20(18.5%)

Discussion

Staphylococcus aureus is one of the main pathogens which cause various diseases in humans. Community health has been affected severely from the emergence of resistant strains. The pathogenicity of S. aureus is associated with its ability to produce numerous virulence factors.

It is one of the infectious agents showing high occurrence. MRSA is a term used for *S.aureus* that demonstrated resistant to following antibiotics namely methicillin, oxacillin, and cefoxitin. *S.Aureus* It is reported to be showing increased prevalence in healthcare settings causing different type of infections. The hospital spread of the infection with MRSA has shown been found to be the cause of increased hospital stay leading to a serious disease and mortality thus instigating high cost borne by the patient. [14]

In this study we assessed the antimicrobial susceptibility pattern of S. aureus from clinical samples at HBS general Hospital Islamabad. In our study, (52.3%) of *S.aureus* were isolated from pus samples. (Table II). An earlier study done at Nairobi also presented that most of the pus samples showed growth of *S.aureus*.[11] The high frequency of *S. aureus* isolated in pus may be ascribed to wounds which are exposed and more disposed to infections and poor hygiene. In Iran, a study was done to show the antimicrobial susceptibility pattern of S. aureus strains isolated from hospitalized patients, maximum number of the isolates were found to be detected from blood specimens (29%).[8] In Nigeria, a study showed that the majority of the isolates were from urine specimens (76%).[10] These studies are in compliance with our study in which in blood culture isolated percentage were 12 (11.1 %) and from urine samples was 33(30.6 %).

The numbers of clinical isolates obtained from females (55.5%) were greater than males (44.5%), shown ratio was 1.2:1 (Table I). The occurrence of *S.aureus* infection was more in elderly (> 50 years,). The occurrence was seen in both male and female mounting to 43.5% of total *S.aureus* infection.

Our study showed that MRSA rate in Islamabad region of or study is 40.7%. In a similar study on frequency of MRSA isolates in Kohat during 2012 was 44% while MRSA rate in Peshawar showed an increase of 54% from year 2009 – 2011.[6]

All the MRSA isolates (40.7%) were also multidrug resistant (MDR). Those isolates resistant to Cefoxitin were found to be co-resistant to Chloramphenicol (51.8 %), Penicillin (53.7%) and Cotrimoxazole (59.2%). All these isolates were 92.5% sensitive to imipenem. The *S.aureus* strains showed pronounced resistance against Cotrimoxazole 59.2%, chloramphenicol 51.8% and vancomycin 37%. (Table III)

In the present study 77.7% of the isolates were found to be sensitive to linezolid which is following a study done in Iran 2009. [7] In our study Linezolid are highly effective against MRSA. Tetracycline also have good in vitro efficacy. Because of the incessantly growing occurrence of Vancomycin Intermediate *S. aureus* (VISA), it is imperious to curtail the use of Vancomycin. Certainly, it should only be used for those patients who have shown resistant to other antibiotics proven by culture sensitivity reports. This step would help evade the consequences of the surge of Vancomycin Resistant S. aureus (VRSA) in our settings. Tigecycline was 100% in a study in Iran.[15,16] Oral use of linezolid and tetracycline should be encouraged as this would result in can allow shorter stay in hospitals.[8]

Conclusion

Resistance to antibiotics has undoubtedly risen to extremely high levels. Certainly, the antibiotics should be prescribed cautiously for the treatment on the basis of results of culture reports infections. This would help elude the consequences of the increase of Resistant *S. aureus* in our settings. Good hospital infection control measures prove to be the main stay against these infections because antibiotics can never be an effective alternate to good medical practice.

Recommendations

When *S.aureus* infections is suspected it is vital to send appropriate specimens for culture and sensitivity. Studies on epidemiology of multiple drug resistant S. aureus and MRSA is the need of the day Resistance genes should be evaluated in community and hospital strains

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Authors Contribution:

[1,3]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work & Final approval of the version to be published

[2,4,5] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

OPEN ACCESS

Effect of Lengthy Working Hours on Health and Lifestyle of Trainee Doctors in Pakistan

About the Author(s)

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Abstract

Background: The medical trainees are the back bone of health care system in Pakistan but due to mismanagement on the part of administration these doctors have to work tremendously long shifts causing sleep deprivation which results in many problems related to psychological, cardiovascular, GIT system; immunity and burn-out syndrome. This study was not conducted in our setup, so we conducted this research to draw attention of concerned authorities.

Objective: To assess the change in health status and lifestyle of trainee doctors in relation to lengthy working hours.

Methodology: This cross-sectional descriptive study was conducted at Rawalpindi medical university & allied hospitals in a period of 6 months. Data was collected by giving questionnaires to 240 house officers and postgraduate trainees, of Allied Hospitals of Rawalpindi Medical College, selected through simple random sampling. The data obtained was statistically analyzed using SPSS version 21.

Results: Out of the 240 doctors, majority (56%) had more than 100 working hours in a week. 82% reported irritability in their behavior during training which affected their family in 51% cases and patients in 39% cases. 87% reported change in their health status during training. 45% doctors slept for <6 hours duration and 24.6% had depression. Routine life complaints such as fatigue and headaches were reported by more than 30% doctors and were found to be more prevalent in those who had longer working hours, UTI was experienced by 31%, severe GIT infections by 39%.

Conclusion: Most of the trainee doctors with lengthy working hours noticed change in daily routine life since the start of job & reported different health related complaints especially GIT infections and UTI.

Keywords: Lengthy working hours, lifestyle, trainee doctors

Conflict of Interest: None **Funding Source**: None

Introduction

Medical residency is an enormously strenuous and time-exhaustive process. Medical trainees spend a lot of time in hospitals and neglect their health to benefit their patients and to increase their medical training,[1,2] which may be damaging to their psychological, physical and social health.[3] Extensive working hours in the medical arena have been connected with weariness,[4] sleep deficiency[5] and declined quality of life.[6,7] For residents, these working environments can intensify the risk of health matters such as obesity, diabetes and cardiovascular disease.[8] More than 60% of trainee doctors in the Medical University of Manitoba facing health problems due to their long working hours duties.[9] These issues not only disturb the health status of the doctors but also compromise their quality of communications with patients and family,[9] and escalates the level of medical errors.[10]

Pakistani health care professionals (HCPs) have a high prevalence of stress owing to work-life imbalance, economic uncertainty, too much dealing with death and dying and every so often hostility.[11] Previous data has revealed that these works linked stressors push them to so many psychopathologies such as anxiety, depression, poor sleep quality and suicidal ideation [12, 13] Moreover, healthcare professional's particularly doctors also account poor lifestyle, dietary and workout habits.[14]

These long-term practices lead to inadequate health practices because a physician's ability to provide health education and counselling is determined by his or her own healthy habits. In addition, patients are expected to practice lifestyles that they perceive from their physicians [15, 16]

Recognizing this, we conducted this cross-sectional survey to assess well-being and lifestyle of Health Care Professionals and to draw the attention of masses

towards it so that duty hours of these trainees should be adjusted to improve their health as well as professional approach.

Methodology

It was a descriptive Cross-sectional survey conducted on house officers and postgraduate trainees of allied hospitals of RMC. Sample size 240 calculated by WHO sample size calculator[17] and completed in 6-months duration. A simple random sampling technique was used. List of all HOs and PGTs took from admin office along with date of joining, those who were joined less than 3 months duration were excluded and then participants were selected through simple random sampling & data was collected by a structured self-administered questionnaire and analyzed on a computer using SPSS version 21. All trainee doctors with lengthy working hours (more than 8 hours per day) including House Officers and PGTs were included. Recently inducted Trainees with less than 3 months of duration were excluded from the study.

Results

Almost half of the study participants were female i.e. 51%. Among all doctors 58% were house officers followed by post graduate trainees (42%). Their night duty or long call duration is mentioned in figure 1 while the interval between long calls is depicted in figure 2. The majority (43%) of doctors were in the 7-9 months of training tailed by 10-12 months (20%). A large number of doctors (49%) were having an astonishing number of 80 working hrs. per week. The Source of food taken by doctors on duty is shown in figure 3. More or less (95%) of the doctors were not following their meal timings during duty hours. Most of the trainee doctors with lengthy working hours noticed fatigue (34%) and irritability since the start of the job & later on reported different health related complaints especially GIT infections (40%). A significant difference in the attendance of social gatherings before the start of training (mean=2.62, SD=0.829) than after training (mean=1.51. SD= 0.629) was observed by applying paired t-test where t (98) =11.30, p<0.001. Before conducting the analysis, the assumptions of normally distributed difference scores were examined. The assumption was considered satisfied as the skew and kurtosis levels were estimated at –0.56 and 0.062 respectively, which is less than the maximum allowable values for the t test. When the relationship between walk/exercise before the start of training and after training was analyzed by t-test, significant difference was found at (99) =7.5, p<0.001. The most gruesome problem faced by trainees due to sleep deprivation is shown in fig 4.Sleep duration (61-70hrs/week) was also affected in trainees before (33%) and after the start of training (1%).

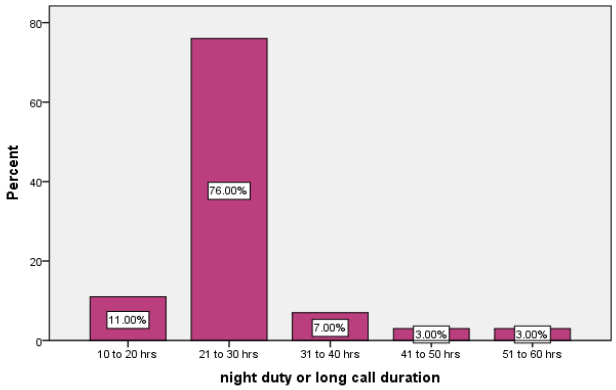

Figure 1. Night duty or long call duration of trainee doctors.

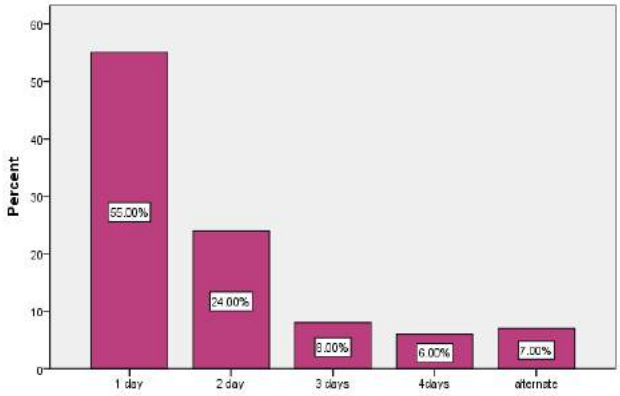

Figure 2. Interval between long calls of trainee doctors

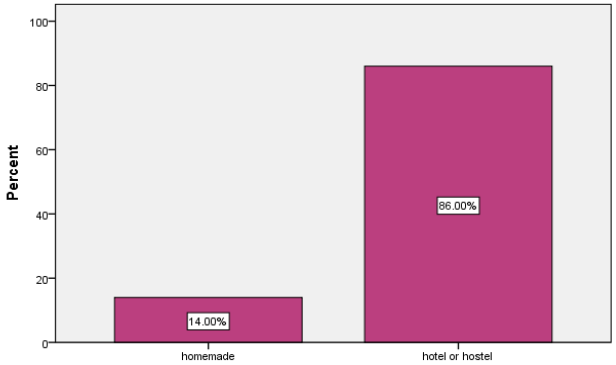

Figure 3: Food mostly taken by the trainee doctors on duty

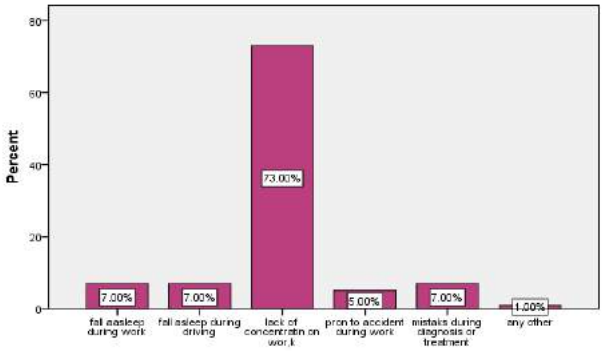

Figure 4. Problems faced by the trainee doctors due to sleep deprivation.

Discussion

In our study all the doctors (100%) reported health issues. Most of the trainee doctors with lengthy working hours noticed fatigue (34%) and irritability since the start of job & later reported different health related complaints especially GIT infections (40%). Similarly, a study on disproportionate was conducted in Germany to see the effects of unplanned heavy working hours on health of doctors. Results depicted that 19 % doctors with prolong working hours suffered ill health complaints. Primarily, they had complaints of fatigue, both mental and physical. Statistical association was significant (p=0.0001). They had fainting feelings with trembling, heaviness in legs, extreme desire to sleep (p=0.0001 to p=0.047) . They were having chocking in throat with chest pain (p=0.0001 to 0. 042). Their bowel system got upset frequently as nausea and loss of weight (p=0.0001 to 0.014). Besides Somatic problems, they were experiencing psychosomatic issues as well. They were irritable most of the time, feeling being very serious, (p=0.0001 to 0.014).[17] The most probable reason for it is when doctors don't get enough time for rest it appears in the form of physical as well as psychological problems.

In our study 37% of doctors reported up to 7 hours of sleep per day. Similar results were seen in another study where it was observed that doctors, getting sleep less than five hours per night (04%) were feeling uneasy, depressed or extremely serious with somatic issues as well like gastro intestinal problems, chest compression etc. Even other group of doctors, having less than seven hours sleep in 24 hours (45%) was having nearly similar issues.[18] It shows that doctors are doing hard work or we can say overwork in all parts of the world. To avoid increased prevalence of hypertension, heart problems like angina, heart attacks, stroke etc, resting and sleeping hours must be adjusted properly. Sedentary life style witting prolonged sitting at work leads to obesity which becomes another risk factor for cardiac problems. Less sleep also leads to impaired concentration.

According to our research, 76% of trainee doctors reported 21-30 hours as their long call duration. In many developed countries, length of working hours at a stretch has been covered under law and is strictly observed. For example, in USA, residents are not allowed to work more than 80 hours in a week. Range of working hours has been set between 40-80 hours in a week. These timings are adjusted depending upon nature of duty in rotations during different specialties of medicine. Very rarely, timings may be stretched up to 136 (out of 168) hours in a week.[19] Other improvement in length of working hours was made according to it, timings were restricted to 16 hours at a stretch for a interns and 28 hours for other trainees in 2011. The main objective of adjusting these timings is to be able to handle critical patients and life threatening condition with complete physical efficiency and peace of mind.[20]

The current study shows a significant difference in the attendance of social gatherings of trainee doctors before and after the training and in 51% cases their family life was also affected.

A similar report shows most physicians work 40-60 hours /week. If time of sleep is taken out from the time left with physicians, very little time is left for "time out of medicine".[21] So the problem of little time for different activities and work life balance is affected badly in the lives of doctors which in turn can lead to anxiety, depression and dissatisfaction among physicians.

In this study it is observed that 73% of doctor's show lack of concentration in work when they are deprived of sleep. Contrary to it, a study conducted by Dr. Govindarajan show that there is not much difference in the performance of surgeons whether they have slept a night before or not.[22] This reason could be because in the study conducted by Dr. Govindarajan all the doctors included were fully licensed and senior doctors, they had the opportunity of cancelling the appointments in the next morning if they felt they are not fit or tiered and their performance could affect the patient care in any way. Whereas in our study, trainee doctors of a Government hospital were included where not only workload is great but also no choice of delaying appointments.

The current study shows that 82% reported irritability in their behavior during training which affected their family in 51% cases and patients in 39% cases and 24.6% showed depression. Another study with similar findings conducted in Canada shows that workload has a positive relationship with stress and negative with satisfaction.[23] Heavy workload seems to invoke cognitive processing of this stressor; continuous workload can induce stress and irritability and later result in job dissatisfaction.

Conclusion

Most of the trainee doctors with lengthy working hours noticed fatigue and irritability since the start of job & reported different health related complaints especially GIT infections. Social life of doctors is also affected and often don't find time for walk or exercise depicting the need of reduction in the duration of working shifts of the doctors & more hospitals should be established to reduce workload.

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Authors Contribution:

[1,2]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work & Final approval of the version to be published

[3,4] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

OPEN ACCESS

Impact of Hyperoxia on Weight, Peptide YY, Lipid Profile and Atherogenic Index in Sprague Dawley Rats

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Abstract

Objective: To determine the impact of hyperoxia on weight, peptide YY (PYY), lipid profile and atherogenic index in Sprague Dawley rats.

Methodology: Experimental, randomized control study was conducted by the Physiology Department, HBS Medical and Dental College, Islamabad in collaboration with National Institute of Health, Islamabad, Pakistan and Islamic International Medical College, Rawalpindi, Pakistan from April 2015-March 2016. Total 40 male Sprague Dawley rats of 2-4 months weighing 250-520 g were included and divided into group A (n=20) exposed to 21% oxygen and group B (n=20) exposed to 30% oxygen for 7 days. Before exposure first weight (g) and blood sample was collected for serum PYY (pg/mL) and serum lipid profile (triglycerides mg/dL, HDL mg/dL, LDL mg/dL, and total cholesterol mg/dL). After exposure second weight and blood sample (serum PYY and lipid profile) was collected. SPSS 21 was used for statistical analysis. Comparisons among groups were analyzed using independent sample t-test. Correlation was done using Pearson's correlation coefficient. In both analyses P value <0.05 was considered significant.

Results: Group B had significantly (P<0.05) increased weight (g) and LDL (mg/dL) levels. Similarly, triglyceride (mg/dL) levels and atherogenic index were also significantly (P<0.01) increased. Significantly (P<0.001) decreased PYY (pg/mL) levels and significantly (P<0.05) decreased HDL (mg/dL) levels were also observed in group B as compared with group A.

Conclusion: Hyperoxia decreases PYY levels causing an increase in appetite leading to an increase in weight, blood lipids (triglycerides and LDL) and atherogenic index. Therefore, hyperoxia may not be useful for treating obesity and cardiovascular disease.

Key Words: Hyperoxia. Weight. Peptide YY. Serum lipids.

Conflict of Interest: None **Funding Source**: None

Introduction

As populations become more urban and incomes rise, the more traditional diets that were high in complex carbohydrates and fiber have been replaced by diets high in fat.[1] This excessive energy intake in the form of food is the main reason for the rise of obesity.[2] The accumulated energy is stored in adipocytes, mainly in the form of triglycerides, leading to an increase in their size ultimately increasing the overall the weight of the body.[3] Alterations in blood lipids such as elevated levels of triglycerides, low density lipoproteins (LDL) and total cholesterol, as well as decreased levels of high density lipoproteins (HDL) are also seen in obese individuals and pose a risk for the development of cardiovascular disease.[4]

Recent research has identified a number of circulating hormones that play a vital role in controlling appetite, ultimately reducing the weight of the body.[5] One such hormone is Peptide YY (PYY), a short 36 amino acid protein, released from the intestines and increases satiety leading to a decreased intake of food.[6] Although, the role of exogenous PYY in reducing weight has frequently been studied more research needs to be carried out on raising endogenously produced PYY as its levels have shown to be low in obese individuals.[7]

Oxygen, the colorless and odorless gas having is widely used for the treatment of various medical conditions.[8] Exposure to low levels of hyperoxia, produce small amounts of reactive oxygen species that have been shown to play an important role in the body such as signaling molecules that regulate various cellular processes and gene expression.[9] On the other hand,

exposure to a high level of hyperoxia for extensive periods causes the production of excessive reactive oxygen species which damage cells through the process of lipid peroxidation.[10]

Research has been carried out to observe how hypoxia affects PYY levels in humans which has shown that exposure to hypoxia decreases serum PYY levels, and as the oxygen concentration is gradually raised back to normoxia (i.e. 21% oxygen) the serum PYY levels have been shown to increase.[11] Studies showing the effect of hyperoxia on PYY levels, especially as a potential treatment for obesity, still needs to be explored. The aim of our study was to see whether exposure to a low level of hyperoxia of 30% could be used as a treatment for obesity and cardiovascular disease by raising endogenous PYY levels, ultimately decreasing the appetite leading to a reduction in weight and serum lipids (triglycerides, LDL and total cholesterol).

Methodology

The research was conducted by the Department of Physiology, HBS Medical and Dental College, Islamabad in collaboration with the Animal house at National Institute of Health, Islamabad, Pakistan and Islamic International Medical College, Rawalpindi, Pakistan from April 2015 to March 2016. It was an experimental, randomized control study. A total of 40 male Sprague Dawley rats of age 2-4 months and weight 250-520 g were included and randomly divided into two groups of 20 rats each: control group A that was exposed to an oxygen concentration of 21% and group B that was exposed to an oxygen concentration of 30%.[12] The rats were allowed to acclimatize to the Animal house environment for 7 days where they were provided food and water ad libitum. The first sample was taken on the morning of day 8 by placing each rat of group A and B individually in a jar containing cotton soaked in chloroform to anesthetize it after which its weight (g) was recorded using a weighing machine (TS200 electronic compact scale, Jiangyin Ditai electronic technology Co. Ltd., China). Blood was drawn via intra-cardiac sampling and collected in labeled gel tubes, protected from light and contamination and kept in a laboratory ice box at 2-8^{o}C until shifted to the laboratory where they were tested for serum PYY (pg/mL) levels using an Enzyme-linked Immunosorbent Assay (ELISA) kit (CUSABIO Biotech Co. Ltd., China) and serum lipid profile (triglycerides mg/dL, HDL mg/dL, LDL mg/dL and total cholesterol mg/dL) using a Merck kit (DiaSys Diagnostic Systems GmbH, Germany). Two transparent plastic chambers of dimensions 1.22 m x 0.72 m x 0.72 m were made. The chamber for group A was not made air tight by keeping the upper two sides open allowing fresh air of a 21% oxygen concentration to enter freely, whereas the chamber for group B was made air tight with only two holes: an inlet for oxygen to enter and an outlet for air to exit. Three oxygen cylinders of 4.5 m^3, 3.4 m^3 and 3.4 m^3 and two nitrogen cylinders of 6.8 m^3 were used. Two flow meters (Richu Medical Regulator YR-88E, Ningbo Beilun DB Marine Co. Ltd., China) were used, one attached to an oxygen cylinder and one to a nitrogen cylinder after which they were connected using a T-tube to allow mixture of both gases before supplying the group B rats.[13] To monitor the oxygen concentration an oxygen sensor (CY-12C portable oxygen concentration tester, CLEVER Co. Ltd., China) was also attached in the chamber for group B rats.

The rats were given 2 weeks for replenishing their blood volume to normal levels[14] after which the experiment was initiated. An oxygen to nitrogen ratio of 3:7 was achieved by adjusting the flow rate for oxygen between 1-2 L/min and that for nitrogen between 3-4 L/min ultimately resulting in oxygen concentration of 30 $\pm$ 1% in chamber for group B rats.[13] When a value of 31% on the oxygen sensor was reached the flow rates were readjusted to bring it back to a 30%. The required air humidity was provided by the water present in the flow meters and these conditions were kept for 24 hours for 7 consecutive days[15] except for two situations where the experiment was stopped for no more than 10 min: first for refilling gas cylinders and second for supplying food and water and to clean trays for waste matter. After the 7 days of intervention, second sample for weight, serum PYY and serum lipid profile was collected on the morning of day 8 in a similar way to that of the first sample.

The labeled gel tubes containing the blood samples were centrifuged in a centrifuge machine (EBA-20 small centrifuge, Andreas Hettich GmbH & Co. KG) for 15 min at 3000 rpm. To measure serum PYY (pg/mL) the quantitative Enzyme-linked Immunosorbent Assay (ELISA) method was used. To measure serum triglyceride (mg/dL) the glycerol-3-phophate oxidase peroxidase (GPO-POD) calorimetric method was used. To measure serum HDL and LDL (mg/dL) the enzymatic calorimeter accelerator selective detergent end point assay was used. The serum total cholesterol (mg/dL) was measured using the Cholesterol Oxidase Phenol Ampyrone (CHOD-PAP) enzymatic quantitative calorimetric method. Statistical Package for Social Sciences version 21 (SPSS 21) was used for statistical analysis and results were documented as mean $\pm$ SEM. Comparisons among the two groups was analyzed using the independent sample t-test and Pearson's correlation coefficient was used for correlations among the variables. For both analyses a P value of <0.05 was considered significant.

Results

The weight of group B rats (309.08 $\pm$ 10.71 g) was significantly higher (P=0.043) than the weight of group A rats (283.75 $\pm$ 5.20 g) as shown in Figure 1. On comparison the serum PYY levels of the group B rats was 14.62 $\pm$ 6.14 pg/mL which was significantly lower (P<0.001) than those of the group A rats (256.87 $\pm$ 25.48 pg/mL) as shown in Figure 2. The rats of group B had serum triglyceride levels of 35.53 $\pm$ 1.51 mg/dL which were significantly raised (P=0.01) as compared to the

group A rats (30.00 ± 1.34 mg/dL). Among the two groups the serum HDL levels of the rats in group B (15.47 ± 0.67 mg/dL) were significantly lower (P=0.028) from those of the rats in group A of 17.39 ± 0.49 mg/dL. Between the two groups the serum LDL levels of the rats in group B were 8.63 ± 0.45 mg/dL which were significantly higher (P=0.020) than the LDL levels of group A rats (7.06 ± 0.46 mg/dL). Mean ± SEM of serum lipid levels (triglycerides mg/dL, HDL mg/dL, LDL mg/dL and total cholesterol mg/dL) for the two groups of male Sprague Dawley rats exposed to different concentrations of oxygen.is shown in Figure 3. On calculating the atherogenic index (total cholesterol/HDL ratio), group B rats had a significantly higher (P=0.007) value of 2.38 ± 0.04 as compared to that of the group A rats (2.22 ± 0.04).

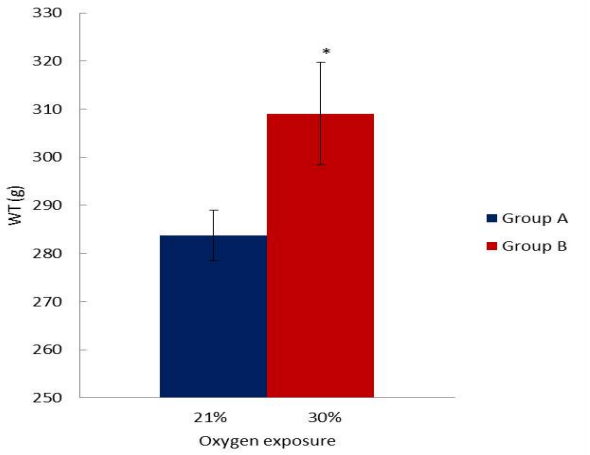

Figure I. Mean ± SEM of weight (g) for the two groups of male Sprague Dawley rats exposed to different concentrations of oxygen. Weight (WT)
** = P<0.05 (value vs corresponding control)*

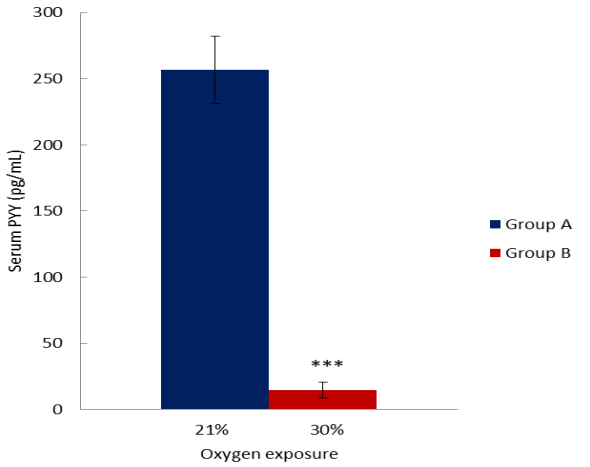

Figure 2. Mean ± SEM of serum PYY (pg/mL) levels for the two groups of male Sprague Dawley rats exposed to different concentrations of oxygen. Peptide YY (PYY)
**** = P<0.001 (value vs corresponding control)*

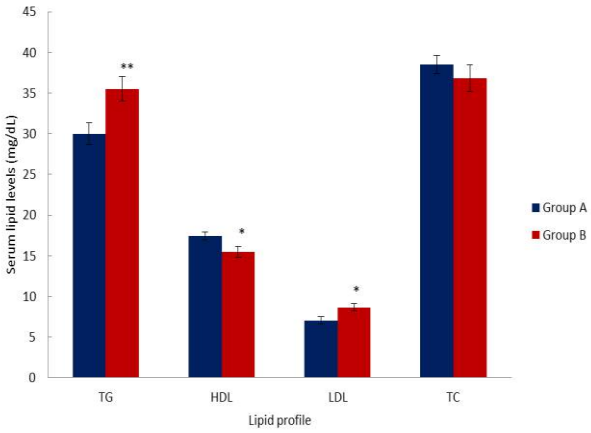

Figure 3. Mean ± SEM of serum lipid levels (triglycerides mg/dL, HDL mg/dL, LDL mg/dL and total cholesterol mg/dL) for the two groups of male Sprague Dawley rats exposed to different concentrations of oxygen. Triglycerides (TG), High density lipoproteins (HDL), Low density lipoproteins (LDL), Total cholesterol (TC)
** = P<0.05 (value vs corresponding control)*
*** = P<0.01 (value vs corresponding control)*

Discussion

In the study carried out by Wasse et al, to explore how rest and exercise in a hypoxic environment influenced PYY levels in 10 male volunteers, it was concluded that the levels of serum PYY decreased on exposure to hypoxia and increased as the oxygen concentration was increased back towards normoxia.[11] Our present study was aimed to see whether this pattern of increase in serum PYY levels was consistent after increasing the oxygen concentration beyond that of a normoxic state. However, according to our results, a 7-day exposure to an oxygen concentration of 30% resulted in a significant decrease in serum PYY levels demonstrating that both hypoxia, as shown by Wasse et al (2011), and hyperoxia, as shown by the current study, decrease serum PYY levels.[11]

The present findings also showed a significant increase in weight on exposure to hyperoxia which was in accordance with the study conducted out by Lakani et al., (2012) who observed the effects of hypoxia, normoxia and hyperoxia on 81 great sturgeon Huso huso fish and concluded that the group exposed to hyperoxia led to the greatest weight gain.[16] The significantly increased weight seen in our study could have been due to an increased appetite caused by the significant decrease in serum PYY levels which is supported by the research carried out by Guo et al., (2006) who showed that fasting serum PYY levels have a negative correlation with weight.[17] However, our results did not show a significant correlation of serum PYY levels with weight.

Fevolden et al., (2002) states that cortisol is the most commonly measured indicator of stress that provides a

good reflection of the severity and duration of the stress response.[18] High cortisol levels stimulate appetite and weight gain as well.[19] Therefore, in our research an exposure to hyperoxia could have led to oxidative stress thus increasing the levels of serum cortisol in the rats causing an increase in their appetite resulting in the weight gain.

Another hormone, ghrelin, could have also been at play as shown in the study carried out by Batterham et al., (2003) to investigate the resistance of PYY in 12 obese subjects in which it was concluded that PYY infusions significantly decreased the levels of ghrelin.[20] The study conducted by Wren et al., (2001) on 9 volunteers showed that ghrelin increases appetite and food intake.[21]

Therefore, maybe the decreased levels of serum PYY in our study could have led to increased levels of ghrelin which ultimately increased the appetite leading to more calorie consumption resulting in the increased weight.

The significant increases in serum triglycerides and LDL levels as seen in our present study are in coherence with the results observed in the study carried out by Tsuneyama et al., (2011) to see the advantages and disadvantages of hyperbaric oxygen treatment in 19 male mice with obesity hyperlipidemia and steatohepatitis, and they concluded that the mice that underwent hyperbaric oxygen therapy had increased serum triglyceride and LDL levels. The underlying mechanism that they suggested was that the lipids may have migrated into the blood from liver cells as hepatocelluar damage was also reported in their study which may have occurred due to oxidative stress and lipid peroxidation hence leading to cell death and causing the release of free fatty acids and triglycerides into the blood from the damaged cells.[22]

The significant weight gain observed in our study could have also led to the significant increase in serum triglyceride levels as supported by the study conducted by del Mar Bibiloni et al, (2015) on 451 2–10 year-old northern Mexican children which determined the levels of serum lipids and prevalence of dyslipidemia. In their research it was deduced that the majority of subjects who had an increased body weight also had a significantly high serum triglyceride level.[23] In the present study we also observed a significant increase in serum LDL levels along with a significant decrease in serum HDL levels which could have also been related to the increase in weight as supported by the cohort study carried out by Horta et al (2009) on newborn males in Pelotas, Southern Brazil who were followed up till the age of 18 years. Their findings showed that those subjects who gained weight throughout the 18-year period also showed significant high serum LDL levels and significant low serum HDL levels.[24]

According to our results there was no significant change in total cholesterol levels, however there was a significant decrease in serum HDL levels which ultimately led to a significant increase in the atherogenic index as supported by the study carried out by Lemieux et al., (2001) to determine the difference in significance between the total cholesterol/HDL ratio and the LDL/HDL ratio as indices of ischemic heart disease risk in middle aged men. They demonstrated that a significant and high atherogenic index is associated with a high risk of ischemic heart disease.[25] This shows that in our present study a significantly high atherogenic index indicates that exposure to even a low level of hyperoxia as that of 30% can lead to ischemic heart disease.

One limitation of our study was that we did not measure the change in adipose tissue mass or hormones such as cortisol and ghrelin which could have helped in clearing the picture of the underlying mechanisms involved.

Conclusion

The results of the present study conclude that hyperoxia decreases serum PYY levels causing an increase in appetite leading to an increase in weight and serum lipid levels (triglycerides and LDL). Therefore, hyperoxia is not a useful option for treating obesity and may be a precipitating factor for the development of cardiovascular diseases.

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Authors Contribution:

[1,3]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work & Final approval of the version to be published

[2,5,6] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

OPEN ACCESS

A Nonsense Mutation Causing Autosomal Recessive Congenital Atricia with Papular Lesion in Pakistani Population

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Abstract

Hereditary atrichia with papular lesions is a rare form of irreversible alopecia with autosomal recessive mode of inheritance. In this form of Alopecia patients exhibits total loss of scalp and body hair loss soon after birth with the development of papular lesions of keratin-filled cysts over the body. Previously several studies have reported sequence variants in the human hairless (HR) gene as the underlying cause of this disorder. In this research study, Sanger sequencing of the gene HR revealed a nonsense mutation p. Cys690* in the family.

Keywords: Missense, autosomal recessive hypotrichosis.

Conflict of Interest: None **Funding Source**: None

Introduction

Hypotrichosis is a heterogeneous group of human hair loss disorder affecting scalp, trunk and rest of the body parts in both sexes. This condition has been reported in both isolated (non-syndromic) and syndromic forms of disorders, which segregate in autosomal recessive as well as autosomal dominant pattern of inheritance. Autosomal recessive forms of isolated hypotrichosis include the most widely reported atrichia with papular lesions (APL), which occurs due to mutations in the hairless gene (*HR*). This condition represents an irreversible form of complete hair loss on the scalp and rest of the body parts followed by appearance of keratin filled cysts (papules) on the skin.[1,2] In the last few years three clinically similar forms of localized autosomal recessive hypotrichosis (LAH1-3), characterized by sparse scalp and body hair, have been mapped on different human chromosomes and the disease causing genes (*DSG4*, *LIPH*, *LPAR6*) have been identified.[3-9]

Atrichia with papular lesion (APL, OMIM 209500) is a rare autosomal recessive hair loss disorder with clinical manifestations of complete absence of hair from scalp and other body parts along with appearance of keratinous papules on the skin.[1,10] APL has been mapped on chromosome 8p21.1.[2,11] Subsequently, many reports described pathogenic variations in the hairless gene (*HR*, OMIM 602302) causing underlying disease pathology The gene *HR* comprised of 19 exons spanning 14 kb genomic distance on chromosome 8p21.1.[2] Hr mRNA is present throughout hair cycle[12] but hair follicles involved in actively growing hair do not contain detectable HR protein. Fifty-one mutations in the HR gene have been reported in families around the world (HGMD, 2014).

In the present study we enrolled a highly consanguineous Pakistani family segregating autosomal recessive hair loss disorder. Linkage analysis confirmed linkage to *HR* gene at chromosome 8p21.1. Mutational analysis through DNA Sanger sequencing revealed a nonsense variant (c.2070C>A; p.Cys690*).

Methodology

Family history: A consanguineous family presented in (Figure 1), segregating autosomal recessive type of hypotrichosis belonging from a remote village of Murree Punjab province of Pakistan. The patients of this family were physically examined by dermatologists of government hospitals. Whole blood samples (4 CC) of all the available patients (IV-4, IV-5, and IV-6) and healthy siblings (IV-2, IV-3) along with parents (III-1 and III-2) were collected in 5 ml K-EDTA tube (BD international).

DNA extraction and genotyping: DNA from peripheral blood samples was extracted using (Gen-EluteTM Blood Genomic DNA Kit; Sigma–Aldrich, St Louis, MO, USA). Concentration of the DNA was quantified by measuring optical density at 260 nm, and samples were diluted to 40–50 ng/lL DNA.

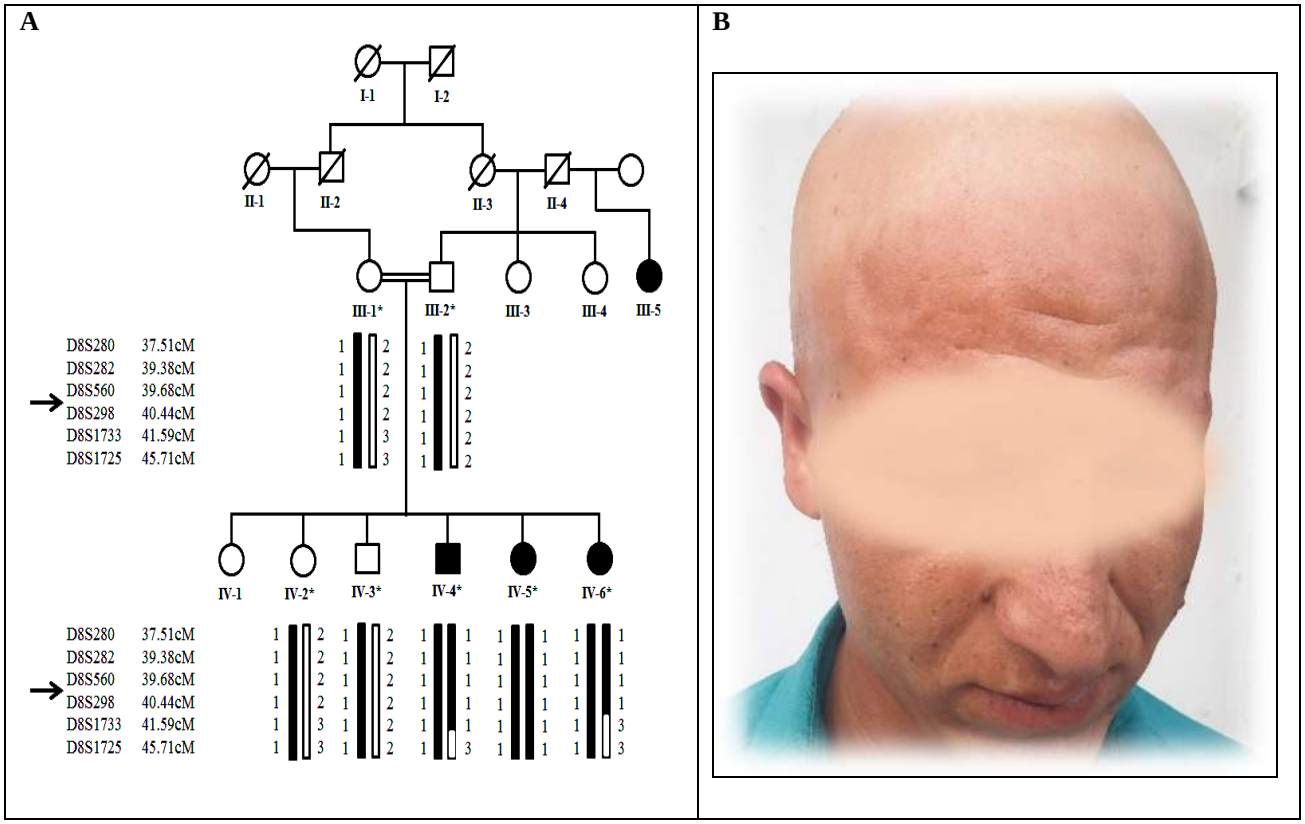

Figure 1: (A) Haplotype analysis of the family segregating APL. For each individual, haplotypes of the most closely linked microsatellite markers are shown below the symbol. The genetic positions (centi-Morgan) and arrangement of microsatellite markers is according to Rutgers combined linkage physical map built 36.2 (Matise *et al.*, 2007). (B) Clinical features of patient showing atrichia (Alopecia Totalis).

In order to perform amplification of genomic DNA through polymerase chain reaction (PCR) polymorphic microsatellite DNA markers were used. PCR done was in accordance with a standard procedure in a total volume of 25 µl containing 40 ng genomic DNA, 20 pmol of each primer, 200 mmol/L of each deoxyribonucleotide triphosphate, 1 U of Taq DNA polymerase and 2.5 lL reaction buffer (MBI Fermentas, York, UK). The thermal cycling conditions used were 95 °C for 1 min, followed by 35 cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified products were resolved in 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and genotypes were assigned by visual inspection.

Based on clinical manifestations presented by patients, this family was tested through microsattelite markers highly linked to *HR* gene locus mentioned in Figure 1.

DNA sequencing: After linkage was established to the *HR* gene at chromosome 8p21.1. All the coding and non-coding exons with 5′ UTR and 2′ UTR of the gene *HR* were sequenced in DNA taken from both affected and unaffected family members (Fig. 2). The primer sequences used for amplification of the entire coding region were as reported previously[1] and are available on request. After purification of PCR-amplified products with commercially available kits (Marligen Biosciences, Ijamsville, MD, USA), sequencing was performed (DTCS Quick Start Kit; Beckman Coulter, Fullerton, CA, USA) in accordance with the manufacturer's instructions. Sequence variants were identified via the BIOEDIT.

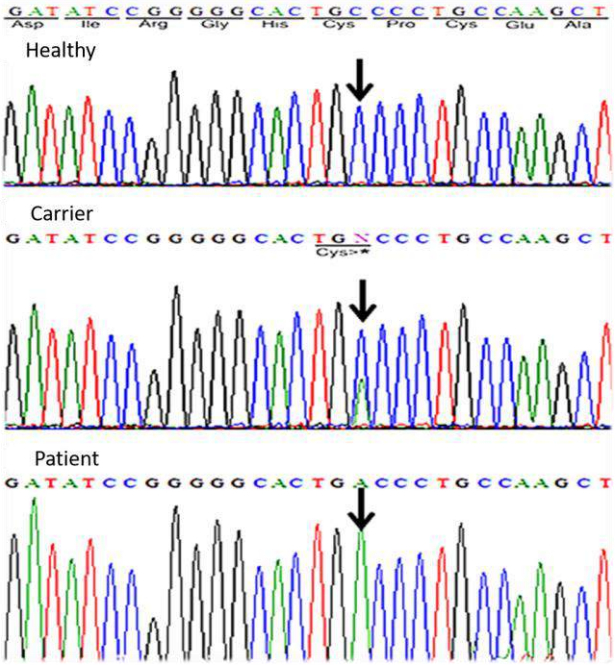

Figure 2: DNA sequence analysis of a nonsense mutation (c.2070C>A; p.Cys690*) in the *HR* gene identified in the family D. The upper panel (Healthy)

represents the nucleotide sequence in the control unaffected individual, the middle panel (Carrier) in the heterozygous carrier, and the lower panel (Patient) in the affected individual. Arrows represent position of the mutation.

Results

Clinical features: All the patients in this family exhibited complete absence of scalp and body hair, and sparse eyebrows and eyelashes. At birth hairs were present on the scalp but never regrew after ritual shaving which is usually performed a week after birth. Papules were observed on scalp, face and other body parts of affected members (Figure 1 B,C). Teeth, nails, sweating and other body organs were normal in affected members of the three families. All affected members were healthy, and physically and mentally active. Heterozygous carrier individuals in the families showed normal hair on scalp and other body parts and were indistinguishable from genetically normal members.

Linkage Analysis DNA Sequence Analysis: Based on the clinical features observed in affected members of the families, hairless (*HR*) was considered as the most potential disease causing factor. Therefore, microsatellite markers (D8S280, D8S282, D8S560, D8S298, D8S1733, D8S1734), flanking the *HR* gene on chromosome 8p21.1, were typed using genomic DNA of both affected and unaffected members of the three families. As expected, linkage in the families was established to the said gene (Figure 1).

To search for the underlying pathogenic variant, all 19 exons, splice sites and a regulatory region (*U2HR*) of the gene *HR* were PCR amplified from genomic DNA of at least two affected and one unaffected member in each family. Once the sequence was identified, the same exon was amplified from other affected and unaffected members of the same family. The primers were designed from intronic sequences of each exon of *HR* gene. The PCR-amplified products were then sequenced following Sanger Cycle Sequencing. DNA Sequence analysis of the gene *HR* was performed using a control reference sequence obtained from the Ensemble database (www.ensembl.org/Homosapiens).

In the selected family, DNA sequence analysis revealed a previously reported protein truncating nonsense mutation (c.2070 C>A, p.Cys690*) in the gene *HR*. This mutation represents substitution of a codon for cysteine with premature stop codon (Figure 2). The sequence variants detected in affected members were present in heterozygous state in obligate carriers of the families.

Discussion

As reported in several cases earlier the family presented in the current study with APL, presented here, showed complete loss of hair on scalp and other parts of the body, Eyebrows and eyelashes were missing as well[1,10,18] (; Sp, 1999, Kim *et al*., 2007; Lee *et al*., 2011; Azeem *et al*., 2011; Wali *et al*., 2006a; Wang *et al*., 2013).

Linkage analysis using microsatellite markers in the selected family showed linkage to Hr gene at chromosome 8p21.1.

Since few year research work was performed by many scientist and it is established that that HR functions as co-repressor for multiple nuclear receptors including vitamin D receptors[13], thyroid hormone receptors (THR)[14] inhibitors of Wnt signaling such as Wise and Soggy[16] (Beaudoin et al., 2005) and retinoic acid receptor-related orphan receptors[15].

Functional studies showed that hairless protein harbors three repression mediating domains (RD1, amino acids 236–450; RD2, amino acids 750–864; RD3, amino acids 864–981), thyroid hormone receptor (TR) interacting domains (TR-ID1, amino acids 816–830; TR-ID2, amino acids 1026–1038), retinoic acid receptor-related orphan receptor (ROR) interacting domain (ROR-ID1, amino acids 586–592; ROR-ID1, 778–782), cystein-rich domain (amino acids 587–712), and JmjC domain (amino acids 946–1175) (Thompson et al. 2009)

Till to date, more than 50 different mutations including deletions, insertions, nonsense, missense and splice-sites in the *HR* gene have been reported causing congenital atrichia. In the present study a nonsense mutation (p.Cys690*) was identified which is predicted to cause nonsense-mediated decay of the mRNA or instability of the truncated protein as reported earlier[17]. The nonsense mutation p.Cys690X identified in the current study lies in the cystein-rich domain. The nonfunctional cysteine-rich domain will lead to total loss of scalp and body hair.

Conclusion

Research findings associated an already reported missense mutation causing human hair loss abnormality. The clinical manifestations observed are consistent with the cases reported earlier. The missense mutations further support the role of *HR* gene in hair follicle development.

Disclosure: This paper is retrieved from PhD Thesis of the principle author submitted in Quaid-I-Azam University in 2015.

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Authors Contribution:

[1,3]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work & Final approval of the version to be published

[2,4,5] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

OPEN ACCESS

Establishment of Reference Intervals for Routine Biochemical Parameters in Adult Residents of Islamabad Territory

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Abstract

Objectives: To estimate reference intervals for ten biochemical parameters in adult healthy blood donors visiting Blood Bank at Shaheed Zulfiqar Ali Bhutto Medical University, Pakistan Institute of Medical Sciences (PIMS) Islamabad and to compare values with the published data.

Methodology: One thousand apparently healthy individuals between the ages of 18-60 years participated in the study. Reference values established using non parametric method.

Results: Mean age was 28.4 years. Mean, median, standard deviation and 2.5th & 97.5th percentiles calculated. Reference values for serum ALT: 10-68 U/L; total Bilirubin: 0.12-1.4 mg/dL; ALP: 51-150 U/L; Urea: 13-40 mg/dL; Creatinine: 0.6-1.3 mg/dL; Uric acid: 3.4-8.2 mg/dL; total Protein: 6.1-8.3 g/dL; Albumin: 3.8-5.3 g/dL; Na: 136-147 mEq/L; and K were 3.1-4.8 mEq/L.

Conclusion: Reference intervals for serum ALT and Albumin revealed significant departure from values provided in reagent kits inserts by the manufacturing company. This difference may be due to environmental, geographical or genetic factors. There is a need to conduct more studies to establish reference values that are true representative of local population.

Keywords: Reference intervals, biochemical parameters, Histopathology.

Conflict of Interest: None **Funding Source**: None

Introduction

The concept of reference values was given by Grasbeck and Saris in 1969.[1] In clinical practice, laboratory test results are always compared with reference values.[2] Reference values are obtained by measurement of an analyte on reference individual selected from a reference sample group[3]. These values comprise central 95% of a healthy population. The limiting values for reference intervals are usually 2.5th and 97.5th percentiles of test results distribution in a reference population.[4] Five percent of healthy individuals have observed values above or below these limits.[4]

Reference intervals should be established according to the guidelines of clinical and laboratory standard institute (CLSI).[5] Reference intervals are influenced by factors like age, sex, nutritional status, geographical location, climate and ethnicity.[6-8] Population should be well defined. It should have resemblance with population under investigation.[5]

In developing countries reference intervals for biochemical parameters are derived from packing inserts of reagent kits manufacturing companies or borrowed from text books which are based on data collected from developed countries.[9]

Most of the clinical labs in Pakistan do not have their own reference intervals.[8] Establishment of population based reference values would help to improve patient's diagnosis, treatment and hence quality of health care.

Methodology

A Cross sectional study was conducted by Department of Chemical Pathology in collaboration with Blood Bank at Shaheed Zulfiqar Ali Bhutto Medical University, Pakistan Institute of Medical Sciences (PIMS) Islamabad from July 2013 to September 2014. One thousand apparently healthy adults belonging to different geographical areas, who visited Blood Bank as blood donors participated in the study. Sampling was started after approval from Hospital Ethics Committee and permission to use blood bank facility. After an informed consent, participants interviewed through a questionnaire. Those with the history of liver, kidney & endocrine disorders, hypertension, recent infections,

tobacco, alcohol and drug abuse were excluded. Body mass index (BMI) calculated for each individual to exclude over weight/ obese subjects. Phlebotomy was performed aseptically. About 450 ml blood was allowed to flow into the blood bag to clear any Citrate Phosphate Dextrose Adenine (CPDA) anticoagulant along the wall of the pilot tube. At the end, 3 ml blood was then sampled from the pilot line into gel vacutainer (Greiner bio-one, Germany). The specimens were labeled with the study number. Post donation screening for HBs Antigen, anti HCV antibodies and anti HIV antibodies was performed on ELISA (Best 2000, SPAIN). Positive cases were excluded from the study. Samples once clotted were centrifuged at 4000 rpm for 5 minutes and transported to the lab in an ice box within 1 hour.

Data collection procedure: Test procedure was performed immediately or within 4 hours of sampling on daily basis for whole duration. Lipemic, hemolysed and icteric samples were rejected. Analysis was performed on Chemistry Analyzer MODULAR P 800 (Roche, Germany) and EASYLYTE PLUS (USA) for electrolytes. Serum ALT and ALP were determined by optimized IFCC method[10,11]. Total Bilirubin analyzed through method developed by Wahlefeldet al.[12] Urea by Talke and Schuberts kinetic UV method.[13] Creatinine analyzed by Jaffe alkaline picrate method.[14-16] Total Protein analyzed by Biuret[17] and Albumin by Bromocresol Green methods.[18] Uric Acid determined by modified Seidles method[19]. Na and K were measured by Indirect Ion Selective Electrode Potentiometer[20]. Reagents were provided by cobas® Roche Diagnostics Germany.[21]

Quality Control samples were run with every batch using Easylyte REF 2378 normal/ abnormal sera (MEDICA USA) for electrolytes and precinorm/ precipath sera (Roche Diagnostics Germany) for rest of the analytes. Daily controls were within ±2SD from the mean. Intra assay CV was within acceptable range.

All data analyzed using SPSS version 16. Frequencies examined as Histogram, mean median, standard deviation and 0.025 & 0.975 fractiles calculated. Reference intervals determined using 2.5th and 97.5th percentiles as lower and upper limits. Both parametric and non-parametric methods were applied. All analytes did not follow normal Gaussian curve due to heterogeneous population. Non parametric statistical method was used to establish reference intervals as per CLSI[5] guideline.

Results

One thousand apparently healthy individuals between the ages of 18-60 years participated in the study. Mean age was 28.4 years. All participants were male. No female participated in the study. Table I describes demographic details of the study participants.

The Participants belonged to different geographical areas of Pakistan. Those residing in Islamabad/ Rawalpindi for last six months were considered as local residents but those who reported to PIMS only for blood donation and residing in their provinces were included in their respective localities.

Frequencies of ten biochemical parameters entered as variables were computed and examined as histogram. Outliers handled by doing range check and Dixon-Reed's Test.[22] Mean, median, standard deviation and reference intervals determined using 2.5th and 97.5th percentiles as lower and upper limits. Whole data was logarithmically transformed and then analyzed.

Both parametric and non-parametric methods were used. Kolmogorov-Smirnov test was applied to the data but normality was rejected for all analytes. Values of coefficient of skewness and kurtosis calculated but the results were not between 1 and -1. Mean, median and mode of all analytes were not similar which also indicated that distribution was not normal.

Reference intervals determined by parametric (mean ±2SD) and non-parametric methods (2.5th and 97.5th percentiles).[23] All parameters did not follow normal Gaussian probability curve due to heterogeneous population. Therefore, non-parametric statistical method was used to establish reference intervals.

Reference intervals for ten biochemical parameters compared with values provided by reagents manufacturing company are shown in Table II.

Reference intervals for serum ALT, ALP, uric acid and albumin were higher than the values provided by reagents manufacturing company. Reference intervals for serum urea, total protein and potassium were lower while values of serum total bilirubin, creatinine and sodium were similar to the values mentioned in literature inserts of reagent kits provided by Roche Diagnostics

Table I: Demographic characteristics of the study participants.

	Total	Islamabad Rawalpindi	Punjab	KPK	Sind	Baluch-istan	AJK	Northern Areas
Total	1000	519	166	181	25	21	62	26
% age	100%	51.9%	16.6%	18.1%	2.5%	2.1%	6.2%	2.6%
Age (years)								
Mean	28.4	28.71	27.63	28.02	29.48	28.81	28.11	27.81
Range	18-60	18-60	18-45	18-52	20-40	18-40	18-53	18-42
Body mass index (BMI)								
Mean	23.39	23.39	23.33	23.49	23.24	23.71	23.39	23.19
Gender								
Male	1000	519	166	181	25	21	62	26

Mannheim, Germany.

The whole data was analyzed collectively as well as separately in subgroups belonging to different geographical areas of Pakistan. Data of subgroups belonging to Islamabad/ Rawalpindi, Punjab and Khyber Pakhtunkhwa (KPK) was analyzed using non-parametric method because the data distribution rejected Kolmogorov-Smirnov test. Data of subgroups belonging to Sind, Baluchistan, Azad Jammu & Kashmir (AJK) and Northern areas was analyzed by Horn's robust technique as the participants were less than recommended number of one hundred and twenty. One-way ANOVA (analysis of variance) test applied to see whether any significant difference lies or not between mean values of subgroups based on geographical distribution. P-value less than 0.05 considered significant. Test results show significant differences in values of ALT and uric acid among the area wise subgroups (p-values 0.006 and 0.022 respectively). Reference intervals of all subgroups are compared in Table III and mean values of different subgroups with their p-values are shown in Table IV.

Discussion

The laboratory test report of a patient is interpreted after comparison with the health associated reference intervals.[24] Most of the labs use reference values

Table II: Reference intervals of ten biochemical parameters of one thousand male participants (18-60 years) by parametric and non-parametric methods compared with values provided by reagents manufacturing company.

Analytes	Unit	Mean	Median	Reference intervals by parametric method	Reference intervals by non-parametric method	Values provided in reagents literature
ALT	U/L	30.12	26.00	9–77	10–68	4–41
T. Bilirubin	mg/dL	0.57	0.50	0.13–1.6	0.12–1.4	0.1–1.2
ALP	U/L	87.77	84.00	45–145	51–150	40–130
Urea	mg/dL	25.61	25.00	12–48	13–40	16–48
Creatinine	mg/dL	0.90	0.90	0.5–1.5	0.6–1.3	0.7–1.2
Uric acid	mg/dL	5.78	5.80	3.3–9.3	3.4–8.2	3.4–7.0
T. Protein	g/dL	7.27	7.30	6.1–8.5	6.1–8.3	6.6–8.7
Albumin	g/dL	4.62	4.60	3.6–5.8	3.9–5.3	3.9–4.9
Sodium	mEq/L	142.05	142.30	136–148	136–147	136–146
Potassium	mEq/L	3.92	3.92	3.1–4.8	3.1–4.8	3.5–5.1

Table III: Reference intervals of ten biochemical parameters of one thousand male participants (18 – 60 years) belonging to different geographical areas of Pakistan.

Analytes	Unit	Combined Data† (n=1000)	Islamabad/ Rawalpindi† (n=519)	Punjab† (n=166)	KPK† (n=181)	Sind* (n=25)	Baluchistan* (n=21)	AJ&K* (n=62)	Northern Areas* (n=26)
ALT	U/L	10–68	11–64	11–70	10–63	8–53	8–68	13–65	12–78
T. Bilirubin	mg/dL	0.12–1.4	0.16–1.5	0.18–1.2	0.17–1.3	0.17–1.3	0.14–1.1	0.18–1.4	0.15–1.2
ALP	U/L	51–150	55–138	53–129	53–135	54–139	46–157	53–136	59–130
Urea	mg/dL	13–40	15–38	16–37	13–38	17–32	16–46	16–38	19–41
Creatinine	mg/dL	0.6–1.3	0.6–1.2	0.6–1.1	0.6–1.1	0.7–1.3	0.6–1.6	0.7–1.1	0.6–1.2
Uric acid	mg/dL	3.4–8.2	3.9–7.8	4.1–7.7	3.7–7.6	4.0–7.2	3.2–8.7	4.2–8.8	4.0–8.0
T. Protein	g/dL	6.1–8.3	6.3–8.1	6.5–8.2	6.4–8.0	6.4–9.0	6.1–8.3	6.3–8.3	6.4–8.3
Albumin	g/dL	3.9–5.3	4.00–5.1	4.1–5.1	4.1–5.2	4.0–5.0	3.7–5.9	4.1–5.4	4.2–5.2
Sodium	mEq/L	136–147	136–146	137–146	137–146	137–146	136–147	137–146	139–147
Potassium	mEq/L	3.1–4.8	3.2–4.6	3.3–4.6	3.3–4.5	3.5–4.7	3.3–4.6	3.4–4.6	3.3–4.3

(n = number) († Reference intervals determined by non-parametric method) (* Reference intervals determined by Horn's robust method)

Table IV: Comparison of mean values of ten biochemical parameters of one thousand male participants (18 – 60 years) belonging to different geographical areas of Pakistan.

Analytes	Unit	Combined Data (n=1000)	Islamabad/ Rawalpindi (n=519)	Punjab (n=166)	KPK (n=181)	Sind (n=25)	Baluchistan (n=21)	AJK (n=62)	Northern Areas (n=26)	p-value
ALT	U/L	30.12	26.19	28.77	24.08	22.08	24.19	29.07	30.20	0.006*
T. Bilirubin	mg/dL	0.57	0.48	0.48	0.49	0.49	0.39	0.51	0.47	0.663
ALP	U/L	87.77	83.51	83.62	85.29	88.51	87.74	84.06	88.73	0.377
Urea	mg/dL	25.61	24.50	24.12	23.75	23.42	26.77	24.60	26.89	0.534
Creatinine	mg/dL	0.90	0.89	0.87	0.86	0.87	0.92	6.88	0.89	0.536
Uric acid	mg/dL	5.78	5.63	5.77	5.49	5.40	5.14	6.08	5.57	0.022*
T. Protein	g/dL	7.27	7.22	7.32	7.25	7.52	7.13	7.18	7.18	0.065
Albumin	g/dL	4.62	4.56	4.62	4.65	4.54	4.66	4.64	4.67	0.246
Sodium	mEq/L	142.05	141.98	142.16	141.97	141.16	141.27	141.95	142.80	0.242
Potassium	mEq/L	3.92	3.89	3.93	3.90	4.06	3.94	3.92	3.76	0.446

provided by reagent kits manufacturing companies or those published in text books that have no detailed informations.[25] Because of biological diversities of a community, it is very difficult to find healthy sample group.[26] This is not feasible to identify, collect and measure enough samples from a large reference population for most of the labs and for this reason, reference values are usually borrowed. Establishing reference intervals is difficult, time consuming and costly exercise.[26] CLSI[5] and IFCC[27] recommend every lab to establish its own reference values because the population serviced is affected by physiological, pathological and analytical factors which are different in different populations.[7,8]

In Pakistan, limited studies have been published on reference values till date.[28] Study conducted by Molla et al at Karachi in 1993, derived reference ranges in clinical chemistry for healthy males and females.[29] Khan F et al have published reference values for common blood chemistry analytes in healthy population of Rawalpindi–Islamabad area in 1997.[30] Sarfaraz L et al in 2013, determined reference intervals for local population in Bahawalpur.[28] More studies are required to establish reference values based on ethnical or geographical distribution of Pakistani population.

In current study, reference intervals of common biochemical parameters have been set for people visiting Pakistan Institute of Medical Sciences (PIMS) Islamabad. IFCC[27] protocols recommend using healthy individuals for derivation of reference intervals. The dilemma is that health status is difficult to validate. This is similar to the problem of trying to prove null hypothesis in statistics.[31] It is also recommended that reference sample group that is randomly selected should be true representative of a reference population.[2] Therefore healthy blood donors who visited blood bank for donation of blood, selected as a sample group of the study.

All participants were male. Due to infrequent blood donation by females, it was not possible to include them in study. Only fifteen female donors donated their blood during study period in emergency situations or with rare blood groups. Female donors were not considered as per CLSI[5] guidelines which recommend a minimum size of one hundred and twenty individuals to establish reference values. According to donor selection criteria of Blood Bank, healthy donors with ages ranging from 18-60 years are allowed to donate blood. So population age was predetermined.

For selection of study participants, strict protocol was followed. Smokers were excluded. Physiological states like exercise and severe stress was ruled out. Donors having obesity, diabetes mellitus, hypertension, liver, kidney & cardiac disorders and those taking pharmacological drugs were excluded after history and examination. Post donation screening for HBs Ag, anti HCV antibodies and anti HIV antibodies was done. Positive specimen for either of the above tests was excluded from the study.

Preanalytical and analytical factors were kept uniform. For derivation of reference intervals, both parametric and non-parametric methods were tried, but non-parametric method using 2.5^{th} and 97.5^{th} percentiles was used to derive reference intervals as per IFCC[27] recommendations.

In this study, mean value for serum ALT was high. Both upper and lower reference values (10-68 U/L) were higher than values provided by kits manufacturing company[21] (4-41 U/L). However, reference interval for ALT was similar to those reported from India[9] (10-68 U/L), Saudi Arabia[32] (0-63 U/L), Kenya[33] (7-61 U/L), Ghana[34] (11.6-53.1 U/L) and Kuwait[35] (3-75 U/L). The results differed from the study values for ALT given by Khan F et al[30] (11-42 U/L) and Sarfaraz L et al[28] (16-44 U/L).

ALT is used as marker of hepatocyte injury and identifies acute or persistent hepatic damage.[34] Wide variations are known to occur in transaminases.[34] Differences are also known to occur between males both whites and non-whites.[34] These differences may be due to genetic, environmental or geographical factors.[6]

Reference values for serum bilirubin were 0.12-1.4 mg/dL. Values were nearly similar to the values provided by the company. Results reported from India[9] (0.3-1.3 mg/dL) and Bahawalpur[28] were (0.3-1.25 mg/dL). Reference values reported by Khan F et al[30] (0.23-0.90 mg/dL) were different from this study. These differences may be due to analytical method used for estimation of serum bilirubin.[30, 12]

Values of serum ALP in current study (51-150 U/L) were higher than the published values by kits manufacturing company[21] (40-130 U/L). Serum ALP results were lower than results of Rawalpindi[30] (126-297 U/L) and Bahawalpur[28] study (130-280 U/L). However, a study conducted by Molla et al[29] have mentioned ALP values for adult males as 19-146 U/L.

Major isoenzymes of ALP in human serum are derived from liver, bone, intestine and placenta. A substantial amount of normal adult activity in serum is of bone origin.[36] Liver ALP activity in healthy sera increases steadily throughout the life.[36] Intestinal ALP component is found in about 25% of normal serum and its concentration increases after meal.[37] Rise of values of ALP in current study may be due to the factor that all samples were taken as random not fasting.[6] Another similar study can be conducted on fasting samples and the effect of meal on reference values of ALP can be determined.

Beside all above mentioned reasons for departure of serum ALT and serum ALP values from published data, the cause may be due to race differences between Pakistani nationals and Western populations.[6] Dufour et al[38] have discussed that the AST activity of African-

American males is 15% higher than that of other races of American males. Serum ALP values of the Blacks are generally 10-15% higher than the Whites.[38]

Values of serum creatinine (0.6-1.3 mg/dL) were also similar to values quoted by kits manufacturing company[21] (0.7-1.2 mg/dL). Values of creatinine depends upon musculoskeletal mass.[6] Males have high creatinine values as compared to females because of greater muscle mass.[39] But in present study, female were not included so this fact was not established. Results of serum creatinine were nearly similar to studies conducted at Rawalpindi[30] (0.6-1.4 mg/dL), Bahawalpur[28] (0.6-1.6 mg/dL), Kuwait[35] (0.7-1.3 mg/dL) and Kenya[33] (0.5-1.4 mg/dL).

Upper limit of serum uric acid in current study (3.4-8.2 mg/dL) was higher than the value given by reagents manufacturing company[21] (3.4-7.0 mg/dL), but the lower limit was similar. Results of serum uric acid were nearly similar to the study from Bahawalpur[28] (3.5-7.39 mg/dL), Saudi Arabia[32] (3.5-8.4 mg/dL) and India[25] (2.6-8.2 mg/dL).

Reference values of serum urea (13-40 mg/dL) were less than the published values in kits inserts[21] (16-48 mg/dL). Similar values were reported from Rawalpindi study[30] (17-38 mg/dL). Study results were different from Bahawalpur[28] (22-51 mg/dL) and India[40] (20-45 mgdL). This difference has been related to associated dehydration in hot climate of their respective areas.[28.40]

Lower and upper limits of serum total proteins (6.1-8.3 g/dL) were lower than the values provided by kits manufacturer[21] (6.6-8.7 g/dL). Serum protein concentration can rise by 10-15 % due to venous stasis during phlebotomy.[32] This artifact was eliminated in current study through continuous flow of blood during blood donation. Serum total protein reference values reported from Rawalpindi[30] (5.8-8.2 g/dL), Bahawalpur[28] (5.5-7.6 g/dL) and Saudia[32] were (6.2-8 g/dL). Geographical, environmental or genetic factors may be the reasons for this difference.[6]

Reference intervals of serum albumin (3.9-5.3 g/dL) were higher than the values provided by kits manufacturer[21] (3.3-4.9 g/dL). Reference values of serum albumin for adult males given by Molla et al in Karachi[29] (3.2-5.3 g/dL) and Saudi study[32] (4.3-5.7 g/dL) were similar to the current study. It is difficult to explain this reason in pathological terms but may be related to the dietary patterns of urban Pakistani nationals with excessive intake of protein rich diet.[41] Serum albumin level of participants was higher on average. This probably indicates the balanced adult male nutritional status of Pakistani people over the past decades which is contrary to the status of females and children who suffer from malnutrition.[42]

Results of serum sodium (136-147 mEq/L) were similar to the reference values given by reagents manufacturer[21] (136-146 mEq/L). Study done by Molla et al in Karachi[29] (134-150 mmol/L) had similar results. Results from Kenya[39] study show higher reference values for serum sodium (136-155 mmol/L).

Values of upper and lower limits of serum potassium (3.1-4.8mEq/L) were lower than values provided by kits manufacturer[21] (3.5-5.1 mEq/L). Reference values of serum potassium given by Molla et al in Karachi[29] (3.5-4.9 mmol/L) and those from Kenya[39] were (3.4-5.4 mmol/L). Dietary factors and hot climate producing dehydration can be possible reasons for this difference.[6]

When results of subgroups compared with each other based on geographical distribution, significant difference revealed in the values of ALT and uric acid (p-values 0.006 and 0.022 respectively).

Physiology of a population is influenced by dietary habits, geography and socio-economic conditions. As a result, measures of normal physiological functions differ from population to population.[34] Therefore reference intervals should be applicable to a definite population instead of reference values established for one population and used for another physiologically different population.[34]

Conclusion

In current study, data for derivation of reference intervals for ten biochemical parameters has been provided. Reference intervals of serum alanine aminotransferase, alkaline phosphatase, uric acid and albumin were higher while reference intervals of serum urea, total protein and potassium were lower than values provided by the reagents manufacturing company. These differences may be due to dietary habits, environmental or genetic factors.[6] Application of these reference intervals would be useful for interpretation of laboratory test results and management of patients in clinical setups.

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[2,5,6] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

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Effect of Nicotine and Camellia Sinensis on Epiphyseal Plate of Developing Chick Embryo

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Abstract

Objective: To observe the effect of nicotine and camellia sinensis on the developing epihyseal plate of chick embryo.
Methodology: The randomized controlled trial study was conducted at Department of Anatomy, Army Medical College (NUST) Rawalpindi in collaboration with Poultry Research Institute, Rawalpindi. The study sample was consisting of four groups each group having ten numbers of eggs. At 48 hrs of incubation, with the help of egg driller a hole was made on blunt end of each egg to inject relevant solutions. After injecting the solutions holes were sealed with paraffin wax. The control group labeled as G1 was given normal saline. Experimental groups, G2 was given green tea extract, G3 was administered with 0.0001% nicotine solution and G4 was administered both 0.0001% nicotine solution and green tea extract.After incubating the eggs for 17 days, the ten eggs from each group were selected. The blunt ends of eggs were opened with the help of forceps and embryos were collected. The embryos were separated from the yolk sac stored in the formalin filled jars for 48 hours. After fixing, samples were placed into decalcifying solution that is 5% Nitric acid for 18 -24 hrs 1. The bone tissue of right and left sides were placed in the duly labelled Tissue cassettes and processed in Leica TP 1020 automatic tissue processor. Paraffin wax was used with melting point range from 40-70°C for embedding. The block was allowed to cool on cold plate.
Results" The observations were done using 40X objective. Control group G1 in comparison to G3 and G4 with mean number of proliferative cells showed p values (0.043) and (0.000) respectively. Experimental groups when compared with each other such as G2 in comparison with G3 and G4 showed result with p value (0.043) and (0.000) respectively. Comparison of G3 and G4 with each other showed significant result with p value (0.000) (Table 1).
Conclusion: From the study it was concluded that green tea tried to neutralize the affect of nicotine but cannot undo the toxicity.

Keywords: Nicotine, Proliferative zone, Camellia sinensis.

Conflict of Interest: None **Funding Source**: None

Introduction

The human being skeleton can be related easily with Avian species, it is the most reasonable to look for the teratogenic effect caused by the administration of different chemicals. The bony skeleton are considered to be strong without the corresponding being very heavy.[2]

Developing Limb morphology occurs from fifteen till thirty fifth by series of normal stages in the development of the chick embryo.[3] Chick Thigh bone ossification starts with endochondral calcification which starts at its centre and extends towards the ends, which remain cartilaginous area and are termed as growth plates. Thigh bone calcification starts at the fifth day of embryonic period[4].The active alkaloid Nicotine is a component found in tobacco, it is related with many diseases reason of increasing oxidative stress. Smoke caused by cigarette is related with many free radicals.[1] Epiphyseal growth plate is a highly organized structure, epiphyseal end is located between epiphysis and diaphysis of long bone. According to developing stages in chick wing and leg, three distinctive cell regions can be morphologically identified. There are three stages, first an outer grouping of loose mesenchymal and myogenic cells, second an osteoprogenitor layer which will later divide to maintain this progenitor layer in a stacked configuration and to produce tightly packed rounded osteoblast. Third region is a core of cartilage where bone is laid down; initially it appears as a layer of Type I collagen-rich osteoid which is mineralized later.[3]

Cigarette smoking and the presence of its different constituents of smoke are significantly responsible for

decreasing the foetal weight at 20th gestational day in mice.[5]

Camellia sinensis is known as a dietary antioxidant that help in reducing oxidative damage caused by macromolecules such as lipids, DNA and other body proteins. Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the ester of epigallocatechin and gallic acid, and is a type of catechin. Catechin is present in green tea but not occur in black tea. During black tea production, the catechins are transformed to theaflavins and thearubigins[6]. In this research the effect of oxidative injury caused by nicotine and how the green tea reverses the toxicity of nicotine is studied.

Methodology

The experiment work was carried out with approval of ethical committee of Army medical college, Rawalpindi. Forty freshly laid fertilized eggs of Fayoumi species were selected at zero hour of incubation. Eggs with unknown time of laying, broken shell, abnormal shape, colour, texture or eggs stored in refrigerator were excluded from study. Simple random sampling technique was used. The study design comprised of four groups, G1, G2, G3 & G4, each comprising of ten eggs. After properly fumigating and clearing of the hatchery, eggs were placed in it. Temperature was maintained at 37.5℃, the relative humidity was kept between 75% and proper ventilation was maintained. Rotation of eggs was done four hourly. After 48 hrs of incubation, holes were made at the blunt end of eggs with the help of egg driller to inject solution.

Under laminar floweggs of each group were injected with their respective solutions in 0.1ml quantitywith the help of insulin gauge needle. In control group G1, 0.1ml normal saline was injected in each egg. For the experimental group G2, 0.1ml of camellia sinensis was injected, solution was prepared from 8 grams of dried leaves of *Camellia sinensis* which were obtained from locally manufactured (Lipton clear green pure) by Unilever Pakistan Limited. Dried tea leaves were soaked in 100 ml of hot distilled water for 15 minutes and then after filtering the solution, the filtrate was reconstituted as 8% solution.[7] For the experimental group G3, quantity of 0.1ml of nicotine solution was injected at 48 hours of incubation into the eggs. The nicotine solution of 0.0001% concentration was prepared from Nicotine stock solution from 1mg/ml Nicotine Vial (purchased from Sigma Aldrich)[8]. In the experimental group G4, two solutions were injected. 0.1ml of 0.0001% Nicotine stock solution was injected through one hole and the second hole was made for the injection of 0.1ml of 8% *camellia sinensis* extract. After injecting the solutions all holes were sealed with paraffin wax, and again eggs were placed in hatchery for incubation. After incubation for 17 days, femur bones were collected. All the samples were fixed by placing it into decalcifying solution that is 5% Nitric acid for 18 -24 hrs.[3] Slides were made which were stained with haematoxylin and eosin for observations at 10X as well as 40X. The numbers of proliferative cells were counted. An ocular micrometer provided with a scale, was used to measure the size of structure under the microscope. Observations were made using objective 10X and 40X. Epiphyseal plates of distal end of femurs were selected, highly organized structure which is present between epiphysis and diaphysis of long bone. In longitudinal section of distal epiphyseal end of femurs, endochondral ossification can be appreciated by precise sequence of events that is proliferation and hypertrophy of chondrocyte (Anderson, 1995).[9] Cell arrangements defined different zones present in epiphyseal plate. Growth plate orienting factor is responsible of orienting cells present in different zones to be arranged either irregularly or in a column wise orientation (Vandereerden *et al*., 2003). [10] One slide per specimen was made which was stained with haematoxylin and eosin for observations at 10X as well as 40X. At 10X alignment of cells, height of proliferative zone can be observed. At 40X mean numbers of cells of proliferative zone were calculated in approximately 15 intact columns per growth plate, and the counts for each cell type were averaged for individual growth plates. Numbers of proliferative cells were counted in approximately 15 intact columns per growth plate, and the counts for each cell type were averaged for individual growth plates. The observations were done using 40X objective. (Figure 2)

SPSS 16 was used for statistical analysis. Mean values and standard deviations were calculated for quantitative variables. One-way analysis of variance (ANOVA) was used to compare mean number of cells in proliferative zone of chick embryo. P value < 0.05 was considered significant.

Figure 1. Eggs being injected with the intervention solutions

Figure 2. Photomicrograph showing 17 days old epiphyseal plate of control group. '1' is flat shaped cells arranged in columns of proliferative zone. H&E stain. X40.

Results

The p value of control group G1 in comparison to G2 was (1.000). Control group G1 in comparison to G3 and G4 showed results with p values (0.043) and (0.000) respectively. Experimental groups when compared with each other such as G2 in comparison with G3 and G4 showed result with p value (0.043) and (0.000) respectively. Comparison of G3 and G4 with each other showed statistically significant result with p value (0.000) (Table I). Mean number of cells of proliferative zone among different groups of 17th day old embryo showing mean value with ± SEM (Table II). Mean number of cells of proliferative zone in control group G1 and experimental group G2 showed mean value 27.800±0.132 and 27.592±0.302 respectively. Whereas mean number of cells of G3and G4 15.340±0.381 and G4 15.542±0.2958 respectively (Table II).

Table 1:Comparison of mean number of cells of proliferative zone among different groups of 17th day old chick embryo.

Dependent Variable	Comparison Between Groups		p value
	Group	Group	
Mean number Of cells of Proliferative Zone	G1	G2	1.000
		G3	0.043
		G4	0.000
	G2	G1	1.000
		G3	0.043
		G4	0.000
	G3	G1	0.043
		G2	0.043
		G4	0.000
	G4	G1	0.000
		G2	0.000
		G3	0.000
p value ≤0.05 statistically significant			

Table II: Mean number of cells of proliferative zone among different groups of 17th day of incubation

Dependent Variable	Groups	Mean ± SEM
Mean number of cells of Proliferative Zone	G1	27.8000 ± 0.13250
	G2	27.5922 ± 0.30231
	G3	15.3400 ± 0.38158
	G4	15.5429 ± 0.29589

Discussion

Smoking is considered to be the one of the possible environmental hazards. In different regions of Asia and Africa it was found nicotine exposure in forms of tobacco consumption is the main risk factors for, lung cancer becoming one of the leading cause of death.[10] In this study effect of nicotine one of the constituent of tobacco seen on the developing femur of chick embryo by looking at the mean number of cells of proliferative zone. Mean number of cells of proliferative zone showed insignificant difference when G1 was compared with G2, as green tea did not significantly affect the growth of skeletal system of chick embryo as compared to the control group. There was statistically significant result when all experimental groups were compared with each other ($p<0.05$).

In the recent study the developing fetus bone mass is related with maternal smoking. The study showed a lower birth weight is related with maternal smoking.[11] As smoking leads to adverse intrauterine conditions as tobacco smoke is related with many toxic substances responsible in destroying the lung parenchyma and causing respiratory distress. [12]

Another study on chick embryonic development showed that abnormal axial rotation was observed in nicotine treated groups.[13] Tobacco exposure has got has got important impact on the damage to the cochlear structure and indicate possible cause of hearing loss and hearing ability development.[14] This idea is supported by another study that is the maturation of glutamatergic inputs in the auditory brain stem impaired by the exposure of nicotine.[15]

During different studies showed that the antioxidant properties of green tea extract on different tissues. Researchers concluded that tea catechins were absorbed from the small intestine and the main component of tea catechin, epigallocatechin gallate, was widely distributed in several tissues including the liver and kidney.[16] It has been proved that antioxidant properties of catechins are related to decreasing growth of free radical, free radical toxic nature, and metal ion chelating properties.[17] The protective effects of green tea extract against nicotine toxicity are due to combination of several different mechanisms, including modulation of expressions of anti-oxidative systems, direct scavenging of free radicals[18], reduction of the levels of several markers of oxidative stress; reduce lipid peroxidation and reduce DNA strand breakage induced by cigarette smoke in

cultured human bronchial cells.[19] The ability of green tea to act as radical scavenger and chelated transitional metals such as iron may be of major significance for the treatment of nicotine-induced pulmonary injury. Another results obtained from the work done on the lung tissue that the injury can be caused by nicotine toxicity and protection of tissues against reactive oxygen species caused by green tea.[20]

Conclusion

So in this study it was concluded that the adverse effect of nicotine on the developing chick embryo tried to overcome with camellia sinensis but the green tea could not undo the oxidative stress caused by the induction of nicotine.

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Authors Contribution:

[1,3]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work & Final approval of the version to be published

[2,4,5] Drafting the work or revising it critically for important intellectual content;

ORIGINAL ARTICLE

OPEN ACCESS

Risk Factors for Osteoarthritis of Knee Joint among Pakistani Population

About the Author(s)

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Abstract

Objective: The aim of the current study was to ascertain the factors associated with knee osteoarthritis in patients presenting to a tertiary care hospital in Islamabad with complaint of knee pain.

Methodology: This was a cross sectional descriptive study conducted at HBS General Hospital, Islamabad from January to September 2019. All patients presenting to the OPD with complaint of knee pain meeting the inclusion and exclusion criteria were enrolled in the study. Demographic and clinical details were recorded and data was analyzed in SPSS.

Results: A total of 110 patients of knee osteoarthritis were included in the study. 74.5% (n=82) were females and 25.5% (n=28) were males. The mean age of the sample was 56.7 years. More than half of the study sample (n=65, 59.1%) were farmers or field workers. The average duration of disease was 6.4 years. A majority of the patients (n=82, 74.5%) had grade-3 and grade-4 radiological changes at time of presentation.

Conclusion Osteoarthritis of the knee is more common in females belonging to rural areas. Patients generally consult doctors when disease has reached an advanced stage.

Keywords: Osteoarthritis; Risk factors.

Conflict of Interest: None **Funding Source**: None

Introduction

Osteoarthritis (OA) of the knee is a joint disorder that results in progressive loss of function, pain, and stiffness.[1] Frequent knee pain affects approximately 25% of adults, and OA is the most common cause of knee pain in people older than 50 years[2] According to the 2010 Global Burden of Diseases study, the burden of OA is increasing most rapidly among musculoskeletal disorders in terms of disability-adjusted life years.[3] Over the coming decades it will impose new challenges on health systems. According to the European League Against Rheumatism's recommendations, plain radiography is used as the gold standard for the assessment of knees with clinical evidence of OA.[4] Previous studies have reported various risk factors associated with knee OA such as older age, female sex, hypertension, raised glucose, obesity, history of knee injury, varus/valgus misalignment, quadriceps muscle strength, and physical workload.[5-8] However most of these studies for risk factors of knee OA have been performed in persons of European origin, so the results cannot be extrapolated to local population. There have been limited studies in Pakistan which have looked at this pertinent area. To reduce the social burden of knee OA it needs to be diagnosed and managed at an early stage for which the epidemiology of the disease needs to be understood and associated demographic factors need to be identified. The current study looked at the factors associated with knee osteoarthritis in patients presenting to a tertiary care hospital in Islamabad.

Methodology

This descriptive cross sectional study was conducted at HBS General Hospital Islamabad from January to September 2019 after ethical approval from the Institutional Review Board. A convenience based sampling technique was used. Patients of both gender, older than 18 years, with non-inflammatory knee pain and no other joint involvement were included in the study. Patients with pregnancy, malignancy, and on steroids were excluded from the study. Furthermore, patients with hyperuricemia, rheumatoid arthritis, SLE, hyperparathyroidism, joint replacement surgery and congenitally deformed joints were also excluded. The participants were informed about the design and aims of the study and consent was taken individually. Demographic and clinical details including age, gender, residence, occupation, BMI, limb length, height, and daily activities were recorded on a pre-designed proforma. Lab investigations comprising blood complete picture, uric acid, lipid profile and blood sugar levels were performed on all patients. Radiographs of both

knees of the patients were done. The severity of the disease was ascertained through radiographs using the following criteria.[9]

Grade 1: Doubtful narrowing of joint space and possible of osteophytic lipping; Grade 2: Definite osteophytes and possible narrowing of joint space; Grade3: Multiple osteophytes, definite narrowing of joint space and some sclerosis and possible deformity of bone ends; Grade 4: large osteophytes marked narrowing of joint space severe sclerosis and definite deformity of bone ends.

The data was entered into SPSS version 20 and analyzed. The categorical variables were described as frequencies and percentages while the continuous variables were described as means and standard deviations.

Results

110 patients with knee osteoarthritis were included in the study out of which 82 (74.5%) were females while 28 (25.5%) were males (Figure-A). The mean age of the patients was 56.7 years with a standard deviation of 4.2 years. Maximum female patients (41.25%) presented between 40-50 years and 35% of male patients presented between the ages of 50-60 years (Figure-B). The average duration of symptoms in the patients was 6.4 years. 49.1% patients (n=54) belonged to rural areas. 31.8% (n=35) of patients lived on the ground floor while the remaining resided on the first floor or higher. More than half of the study sample (n=65, 59.1%) were farmers or field workers.

The average waist and hip diameter in males was 0.96 while in a female it was 1.05. The average BMI in males was 23.3 kg/m^2 whereas in females average BMI was 26.9 kg/m^2. The right knee was involved in 39 (35.5%), left knee in 47 (42.7%) and both knees in 24 patients (21.8%). Radiological examination of knee joints indicated that 59% of patients (70% of males and 56.3% of females) had grade 3 disease. The most common symptoms included difficulty in standing from squatting position, climbing stairs and offering prayer in standing posture (normal prayer posture). Average blood pressure in males was 153/99 mm Hg while in females was 142/94 mm Hg.

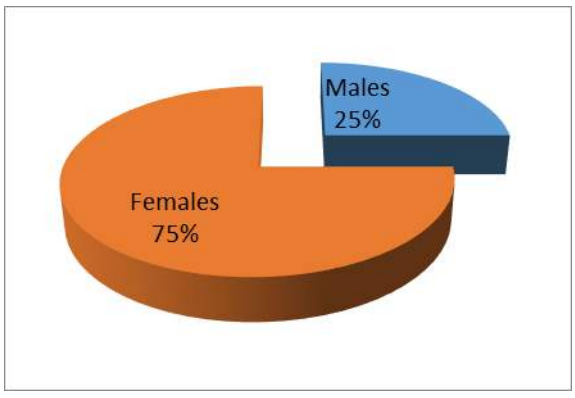

Figure A. Pie chart showing gender distribution of sample

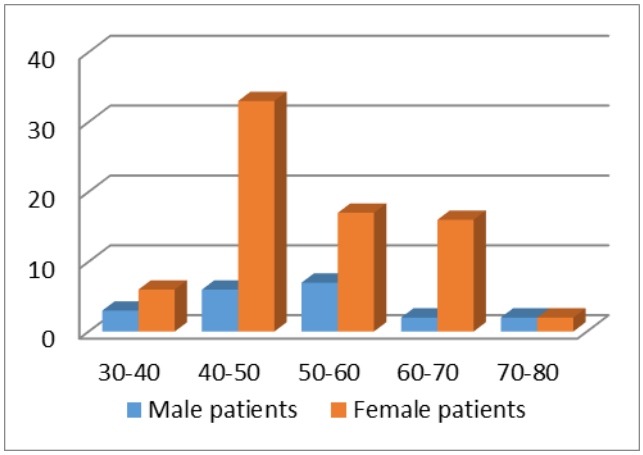

Figure B. Bar chart showing age distribution of sample

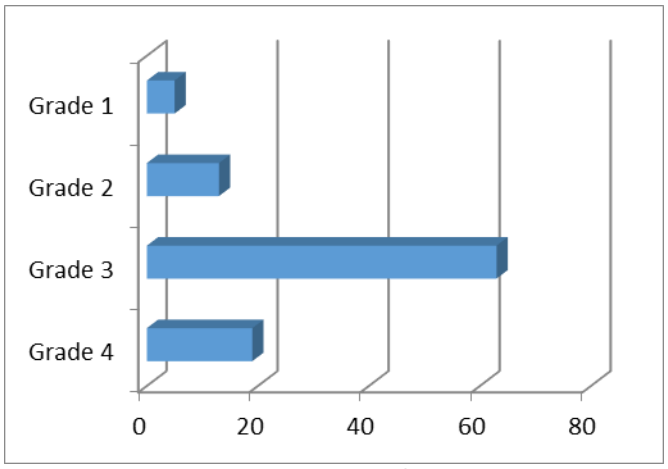

Figure C. Bar chart showing grades of osteoarthritis in the study sample based on radiographic findings

Discussion

Knee osteoarthritis (OA) is a chronic, degenerative disease that increases with age. Knee OA is becoming a great public health problem around the world due to increasing life expectancy, and it is considered the leading cause of disability in the general population in people older than 65. The current study aimed to ascertain the factors associated with osteoarthritis of the knee joint in the local population.

Out of the 110 patients with knee osteoarthritis included in the study, the majority were females (n=82, 74.5%). Increased prevalence of knee osteoarthritis in females as compared to males has been reported in previous literature; internationally as well as locally. A study conducted by Ghaznavi et al in 2017 looked at the pattern of symptomatic and radiographic osteoarthritis in the urban population of Karachi. Their results also showed a predominance of females among OA patients (63.5% vs 26.5%).[10] A number of reasons have been cited in literature as to why females are more prone to osteoarthritis. These include differences in the immune response as well as mechanical burden on knees due to wider hips.[11] The mean age of the patients of our sample was 56.7 years which is again in range with previous studies conducted on the topic. In a study conducted by Lawrence et al to determine the prevalence of

osteoarthritis in the US population, it was seen that the risk of OA almost doubled from the age of 30 to 60 years.[12] Another point to note here is the duration of symptoms before these patients consult health services. On average patients reported having these complaints for more than 6 years before they sought professional help. This was even worse in case of females where this duration increased to 7 years. It has been shown in previous studies that a delay in seeking treatment for OA leads to greater disability and functional loss in later years.[13] Another finding of the study was that people belonging to rural backgrounds and working in fields are more likely to develop OA (59.1% vs. 40.9%). This can be explained by the physical strain their work puts on the knees especially the constant bending and standing up. In a study by Nikolic et al looking at the rural population living in Serbian enclaves in Kosovo found the prevalence to be as high as 84%.[13] The study also showed that 59% patients (70% of males and 56.3% of females) had grade 3 disease on radiographic diagnosis. This again shows a general apathy towards personal health as well as lack of medical facilities at grass root levels due to which early symptoms are ignored by patients and they seek professional help only when disease is significantly advanced and prognosis has significantly deteriorated. In reality, many of these patients were already beyond the conservative management stage and would require major replacement surgery to maintain quality of life in coming years.[14]

Another incidental finding of the study was high blood pressure in a significant number of the patients (n=42, 38.1%). This may be attributed to the prolonged use of NSAIDs in most of these patients; future studies may look at this area in more details.

One of the limitations of the study was that the entire sample was taken from a single institution hence the results may not be generalized broadly.

Conclusion

Osteoarthritis of the knee joint is common in the local female population. Most patients have advanced disease by the time they seek professional help. Rural background and increasing age are other factors associated with knee osteoarthritis in our country.

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Authors Contribution:
[1,2,]Substantial contributions to the conception or design of the work; or the acquisition analysis and Analysis

REVIEW ARTICLE

OPEN ACCESS

Alveolar Osteitis: A Latest Review

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Abstract

Dry socket, also known as fibrinolytic osteitis or Alveolar Osteitis (AO), is amongst the most common complications following tooth exodontia in dentistry. A substantial proportion of research literature is available to alveolar osteitis with reference to its etiology and pathophysiology. Many studies are available for techniques to prevent AO but controversy still exists regarding the actual cause, pathophysiology, methods of prevention and treatment. Review of the concepts and controversies surrounding AO is an aim of this article

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Introduction

"Dry socket" was first described in 1896 by Crawford[1]. Many other terms also been referred to this complication, such as septic socket", "localized osteitis "alveolalgia", alveolar osteitis", necrotic socket", "alveolitis sicca dolorosa", "alveolitis", "localized alveolar osteitis" and "fibrinolytic alveolitis". In spite of acceptance of Birn's theory by many authors, the term fibrinolytic osteitis is no commonly used.[2,3] The "alveolar osteitis" are more commonly used while "Dry socket" is referred as general public term.Approximately there are more than eighteen definitions of AO. The most accepted defines AO as "postoperative pain inside and around the extraction site, which increases in severity at any time between the first and third day after the extraction, accompanied by a partial or total disintegrated blood clot within the alveolar socket with or without halitosis".[2] Sever postoperative pain results in excessive use of medications, repeated hospital visits hence increase financial, psychological and physical burden to the patient while delayed recovery period increased cost to the surgeon as well.[1,2] The previous studies regarding pathogenesis of dry socket are yet not well understood. The studied on AO are still subject to controversies regarding pathophysiology of risk factors and contributing factors. Birn, labeled it as fibrinolytic alveolitis with reference to understanding of the pathophysiology.[6–7]

The pain in empty alveolus is commonly present in all patients with AO [6,8–9]. The other signs and symptoms some time may exaggerate the intensity of AO like radiating pain towards the ear and temporal region[10, 11], maxilla, frontal and ocular regions, infrequent low-grade fever[10, 11], inflammation of the gingival margins, grayish discharge, bare alveolar bone[12,13], ipsilateral regional lymphadenopathy[11], and halitosis.[10, 14] Simple dental extractions reported the incidence of AO has been in the range 0.5% to 5%[15, 16, 17, 18]. In mandibular third molars extraction it varies from 1% to 37.5%.[19, 20] While surgical extractions about 10 times higher incidence are reported. About 95–100% of all cases of AO appear within a week[15] generally AO onset is considered to occur 1–3 day after tooth extraction.[10, 21, 22]

Etiology

Birn suggested that the etiology of AO is an increase in local fibrinolysis leading to disintegration of the clot.[6] The fibrinolysis is the result of plasminogen pathway activation. The activator substances in AO are direct (physiologic) or indirect (non-physiologic).[7] Due to trauma to the alveolar bone cells the direct activators are released while bacterial streptokinase release indirect activators. The fibrinolytic activity is limited to local area because initial absorption of plasminogen into the clot limits the activity of plasmin. The active plasmin is inactivated in the general circulation by antiplasmins.[23]

Birn and many researchers revealed the local differences in the fibrinolytic activity between different body tissues. Higher fibrinolytic activity was observed with bone and uterine tissues, in comparison to, thyroid tissues, heart, kidney, brain, spleen, liver, lung, and skeletal muscle.[24, 25] The factors responsible of triggering fibrinolysis are found to be more ambiguous. The risk factors and contributing factors for development of dry socket has been reviewed by many researchers.

Risk Factors

The risk factors reported are, Systemic Disease, Oral Contraceptives, Smoking, Bacterial Infection, Excessive Irrigation or Curettage of Alveolus, Local Anesthetic with Vasoconstrictors, Bone/Root Fragments Remaining in the Wound and different Flap Design/Use of Suture.

Systemic Disease

AO is linked with many systemic diseases, diabetic and immunocompromised patients are prone to develop AO due to altered healing.[26] The occurrence of AO is controversial in the diseases with pre-existing hypovascularity such as osteonecrosis induced by radiotherapy, cemento-osseous dysplasia, osteopetrosis, paget's disease and other vascular or heamtological disorders.

Oral Contraceptives

Dry socket is commonly associated with oral contraceptives. Therefore, higher incidence of AO is seen in females.[27, 28, 29] Estrogen plays an important role in the fibrinolytic process, Sweet and Butler[30] suggested that higher incidence of AO directly correlates with the increase use of oral contraceptives. It has been reported that indirect activation of fibrinolytic activity cause by oral contraceptives leads to increase in factors II, VII, VIII, X, plasminogen which increases the lysis of blood clot in AO.[31] It has been also found that occurrence of AO enhanced with the increase of estrogen dose in oral contraceptives Catellani et al.[32] To prevent the risk of hormonal cycle's involvement in AO it is suggested for scheduling the elective surgical exodontia.[29]

Smoking

The occurrence of AO has been studied having a dose dependent relationship with the smoking. 4000 surgically removed mandibular third molars, patients who smoked a half-pack of cigarettes a day had developed four- to five-fold increase incidence of AO (12% versus 2.6%) as compared to nonsmokers. AO increased to 40% who smoked on the surgery day as compare to those who smoked a pack per day having more than 20% increase[33]. However the exact mechanism like direct local affect including heat or suction for the increase of AO incidence is not very clear[34]. The introduction of foreign substance by means of smoke fumes could act as a contaminant in the extraction wound[35].

Microbial Manifestation

It has been documented in most studies that bacterial infections are a major risk factor for the generation and growth of AO. The recurrence of AO increases in patients with risk factors such as poor OH[36], preexisting local infections such as pericoronitis as well as periodontitis.[37] The isolation of the causative organisms has been made via cultures. Rozantis et al[38] demonstrated delayed healing of extraction sites after the inoculation of specific microorganisms such as Actinomyces viscosus and Streptococcus mutans, in animal models. Cultures of Treponema denticola, a periodontal disease microorganism, exhibited high plasmin like fibrinolytic projections, according to Nitzan et al[39] Catenalli[40] studied bacterial pyrogens *in vivo* and proposed that they are indirect activators of fibrinolysis.

Excessive Cleansing or Curettage of Socket

Studies have hypothesized that repeated and excessive irrigation of alveolus might hinder the clot formation and that aggressive curettage might also be injurious to the alveolar bone, both of which may lead to the formation of dry socket, however, the literature lacks evidence to certify these claims put forth in the development of AO.

Vasoconstrictors with Local Infiltration

The use of local anesthesia with vasoconstrictors increases the risk of AO. Lehner[41] studied that because of the poor blood supply due to infiltration anesthesia, a temporary ischemia is induced which increases the frequency of AO. However, followed studies suggested that ischemia lasts for one to two hours and is followed by reactive hyperemia, which is a negligible factor in the disintegration of blood clot.[6, 42] Moreover, in a study it was documented that there is no significant difference in the AO of a tooth extracted with infiltration anesthesia versus regional block anesthesia with vasoconstrictor.[34] Therefore, it is currently accepted that local anesthesia with vasoconstrictor has no role in the development of local ischemia which can lead to the formation of AO.

Bony Fragment Remnants in the Wound Site

Chow O and H. Birn have suggested in their studies that bone/root fragments and debris can lead to delayed wound healing, and, consequently aid in the development of AO [2,6]. Simpson, on the other hand, in his study, showed that small bone/root fragments are commonly present after extractions and these fragments do not necessarily cause complications as the epithelium is able to form an external barrier [43].

Design of the Flap and Suture Usage

Some previous literature claims that flap design and the use of sutures affect the development of AO.[27] However, little evidence is found to authenticate such relationship in studies that have recently been conducted.[84] In the absence of any significant evidence, it is practical to assume that these are not major contributing factors.

Contributing Factors

The contributing factors in pathophysiology of AO are, Lack of Operator Experience, Mandibular Third Molars, Patient's Gender, Age, Physical Dislodgement of the Clot, Single Extraction versus Multiple Extractions, Saliva, Surgical Trauma and Difficulty of Surgery

Operator's Inexperience

Operator's inexperience is also considered as a major risk factor for the development of AO. A study carried out by Larsen[44] stated that surgeon's lack of experience during the surgical extraction of third molars can lead to deleterious consequences. Alexander[3] and Oginni et al.[45] have reported an increased incidence in AO carried out by inexperienced surgeons. Henceforth operator's skills and experience should be considered.

Extraction of Mandibular Wisdom Teeth

Many studies have shown the same pattern of increased AO after extraction of third molar.[46, 47] It is a common belief among some authors that increased density of the bone, decreased vascularity and reduced capacity of formation of granulation tissue can lead to formation of AO[46]. However, no evidence could provide a nexus between AO and reduced blood supply. The reason why surgically extracted third molar are prone for the development of AO is due to surgical trauma, and not for their anatomic location.[34]

Patient's Gender

In many studies, it has been stated that female gender is more susceptible for the development of AO, despite the use of oral contraceptive pills. MacGregor[16] reported an increase of 50% occurrence of AO in females as compared to men in his study of 4000 extractions, whereas, Colby had no difference to show in the same scenario and stated that there is no gender association with the development of AO.

Patient's Age

There is little evidence to prove the association of incidence of AO with age. The literature supports the general principle of greater risk associated with old age. Blondeau et al.[49] concluded that surgical removal of impacted mandibular third molars should be carried out well before age of 24 years.

Clot Dislodgement

In a contemporary opinion, there is no corroboration that dislodgement of blood clot caused by manipulation or negative pressure is created by sucking a straw as a major contributor AO.[34]

Single Extraction Compared to Multiple Extractions

Limited data exists indicating a higher prevalence of AO after single extractions as compared to multiple extractions. In one study, AO prevalence was 7.3% following single extractions and 3.4% following multiple extractions.[34] This difference could be a possibility due to the fact that patients with single extractions have less pain as compared to the patients with multiple extractions, whose teeth are damaged drastically.[50] Moreover, multiple extractions involving periodontally diseased teeth may be less traumatic.

Saliva

A few authors have argued that saliva is a risk factor in the development of AO. However, no firm scientific evidence exists to support this claim. Birn found no evidence that saliva plays a role in AO.[51, 52]

Trauma Due to Surgery and Difficulty of Surgery

Many authors agree on the same point that trauma and difficulty while performing surgery can play a significant role in the development of AO.[48] This is because traumatic extractions lead to the production of direct tissue activators following bone marrow inflammation.[34] An increase in incidence of AO is seen by 10-folds in surgical extraction as compared to non-surgical extractions.[26] Lilly et al.[8] stated that surgical extraction inclusive of a flap design and bone removal are more prone towards the development of AO.

Prevention

Numerous techniques are proposed in existing literature for its prevention. However, not a single method has been accepted in law. The most common techniques are discussed as under

Systemic Antibiotics

Systemic antibiotics including penicillins[54, 57], clindamycin[54, 55], erythromycin, and metronidazole[21, 58] are used systemically pre/postoperative, however, the prophylactical use of these antibiotics is debatable due to the emerging strains of resistant bacterias, the occurrence of hypersensitivity associated with these drugs and mainly, the killing of host commensals.[56]

Topical Antibiotics

The use of topical tetracycline has shown to be an auspicious drug amongst other local antibiotics.[59, 60, 62, 64] Foreign body reactions, such as Myospehrulosis, have been reported with the application of petroleum-based tetracycline-hydrocortisone combination.[61, 63, 65] A study reported a nerve dysesthesia six months after mandibular third molar extraction by the use of medications in the socket[63]. The delivery method included powder, suspensions, gauze drain and Gelfoam sponges. It has been also suggested that practically anything into the alveolus, including plain Gelfoam, will result in the improvement in AO symptoms.[10]

Para-Hydroxybenzoic Acid

The prevention of AO by the use of PHBA (para-hydrobenzoic acid), an antifibrinolytic acid has been documented.[66, 68] Alveolar cone has PHBA which includes acetylsalicylic acid and PHBA. A pernyl

success in AO proved good [69, 67], but it is found that it inhibited the bone healing. PHBA has een shown to have satisfactory antimicrobial effects. Local irritation and acute inflammation of the socket is reported with the use of aspirin.[70]

Chlorhexidine

Its stated that use of 0.12% chlorhexidine rinses pre and peri operatively reduces the incidence of AO after third molar extraction.[73, 74] It has been reported that rinsing with chlorhexidine solution before extraction there is 50% reduction of AO.[76] Promising results have been seen with the use of 0.12% chlorhexidine rinses on the day of extraction.[71]

Polylactic Acid

The polylactic acid (PLA), is a biodegradable ester (a clot assisting agent), has been advocated in prevention of AO. PLA helps in the formation of blood clot, granulation and osteoid tissue[53], but few follow-up studies could not support the PLA role in AO rather Complications and incidence of AO were higher with the use of PLA.[77, 78]

Tranexamic Acid

Tranexamic acid (transamin), an antifibrinolytic agent, has been recommended to prevent AO when applied topically or IV after in the extraction.[77] But when compared to a placebo group it did not show a significant reduction in the incidence of AO and local plasminogen inactivation found to be insufficient to cease the appearance of AO.[78]

Steroids

Corticosteroid remained in use to decrease postoperative complications but to prevent development of dry socket no promising results found[79]. Topical application of hydrocortisone and antibiotics remarkably lessens AO especially following the extraction of wisdom molar removal[80]. It is observed the use of steroids alone without any antibiotic combination is not promising.

Eugenol Containing Dressing

Dressings containing eugenol has been very popular for the prevention of AO development.[81] Eugenol causes delay in wound healing and local irritant effect which have made it difficult to conclude if for prevention in dry socket.[83, 82]

Lavage

Butler and Sweet[84] reported that that copious preoperative and intraoperative lavage reduce the incidence of dry socket reported significant reduction in AO, however they found that increase or decrease in the lavage volume do not affect its efficacy.

Aminoacrinide

9-aminoacridine is an antiseptic agent; its effectiveness in reducing the incidence of AO is found to be less effective .[85]

Use of Sterile Gloves

The effect of sterile gloves use instead of clean nonsterile gloves to decrease in the incidence of AO found to be very insignificant.[86, 87]

Management

The most important aim for dry socket management, is to minimize the pain and promote normal healing process. In some cases, where necessary, the systemic use of pain relievers or antibiotics may be used. Intra-alveolar dressings are widely available and used, but with the reference that it might cause delayed healing following extraction.[82] A mixture of 18 different ingredients combines to form a variety of medicaments that are used all around the globe. Alveogyl, a medicament that has been frequently found in the literature, is a combination of butamen (anesthetic), iodophorm (antibacterial) and eugenol (analgesic). The use of alveogyl is reported to have shown results of delayed healing and increased gingival inflammation. The use of ZnO eugenol, fibrin substitutes, whitehead varnish and BIPP are also been shown to be effective in management of AO

Conclusions

Clinically, the post-operative condition of alveolar osteitis is fluctuating and is of debate. The poorly designed previous studies, lack of ideal analysis, statistically biased consists of separate opinions. The etiology being unknown, AO results in varying controversial descriptive definitions and criterions. The initiation of fibrinolytic process appears to be interfacing multiple independent factors in wound healing of AO. There is no single universally accepted method to prevent this complication. Multitude of intra-alveolar medicaments are available on the market however their complications/severe reactions from placed in the socket are rare. The management of this complication should begin with patient education and identifiable risk factors should be informed in detail about this anticipated complication of exodontia. Further well-designed studies based on latest investigations methodologies are necessary to draw firm conclusions of AO treatments.

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CASE REPORT

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Meckel's Diverticulum Presenting as Small Intestinal Obstruction

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Abstract

Meckel's Diverticulum is seen in 2% of the population. It can present variably with hemorrhage, gangrene, perforation, intussusception, ulceration and intestinal obstruction. Sometimes patients present with pain right iliac fossa mimicking acute appendicitis. In those patients with bleeding abnormal gastric mucosal ulcer should be ruled out.

Here we report a 49 yr old lady with pain abdomen. She had persistent vomiting and no stool passage. On examination there were absent bowel sounds and exploratory laparotomy revealed gangrenous perforated Meckel's Diverticulum. So it was excised and intestinal repair was performed. Intestinal obstruction is commonest cause of Meckel's diverticulum in adults.[1]

Keywords: Meckel's Diverticulum, Small bowel obstruction, Gangrene, Perforation.

Introduction

2% of the general population has Meckel's diverticulum since birth as an anomaly. The vitelline or omphalo-mesenteric duct in its most proximal portion having obliteration leads to its formation.[2] There are multiple complications of Meckel's diverticulum such as blood in stool, absolute constipation, ulcer inflammation and black discoloration.[3] Our report is of gangrene and perforation of Meckel's diverticulum that is a rare complication.

Case Presentation

A 49 years lady landed in the emergency department with severe pain abdomen, swelling, vomiting and absolute constipation for 3 days. On clinical examination abdomen was tense, distended with generalized tenderness abdomen. Bowel sounds were hyper-dynamic. Guarding was present in right hemi-abdomen. Her abdominal erect x rays had air fluid levels showing small bowel obstruction. On ultrasound abdomen dilated fluid filled gut loops with inter-loop fluid was seen. Digital rectal examination was unremarkable. All labs were normal except raised leucocytes. Emergency exploratory laparotomy was performed. At laparotomy, there was a loop of ileum stuck due to gangrenous perforated Meckel's diverticulum at the tip leading to small bowel obstruction. Simple diverticulum excision was performed. Its length was 8cm and it was 2.5cm wide. Post op smooth recovery occurred. Inflammation of diverticulum was proved on histology with small area of dead tissue.

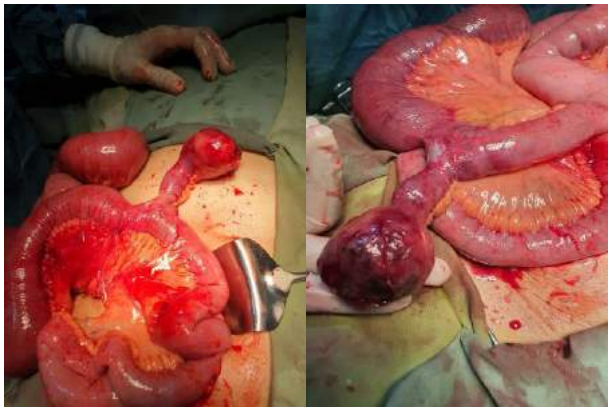

Figure A & B:-Black gangrenous patches on Meckel's Diverticulum with small perforation at broad top marked with broad arrows in figures A & B. There is proximal distended gut marked with small arrow on right side in Figure A.

Discussion

Meckel's diverticulum was discovered by Fabricius Hildanus in 1598 was renamed after Johann Friedrich Meckel in 1809.[6] Shape is like a pouch with all the layers of the intestinal wall. It is usually seen on the border of intestine away from mesentery. Location is around 2 feet proximal to valve of ileum with caecum. They become symptomatic when bigger than 5cm and categorized as giant. Most of patients have them without any symptoms and some have incidental diagnosis during some other procedure. Overall incidence in literature is around 9.2%.[3]

There is 4% chance for a person with this disease to have related complications. One of rarest in children is black discolored diverticulum.[6, 16] Twist around the axis of diverticula is due to bands adherent at the tip, small attachment at intestinal end, malignancy or swelling with redness of area with long pedicle of diverticulum.[6]

Sometimes the patient can be picked as a case of diverticulum because it mimics as other emergency conditions like appendicitis etc. Radiographs and CT scan cannot fully diagnose a case with this disease.[9]

In children perforation is seen in 10% cases[8]. During infection there is pain right iliac fossa, temperature is raised, nausea and features of acute appendicitis.[2] Obstruction of its lumen causes stasis with superadded infection.

Bands at the tip cause adhesions and gut gets stuck in loops[1]. In another study an adhesion was the cause of intestinal obstruction but it was strange that it was presented as partial intestinal obstruction.[10] Perforation of a Meckel's diverticulum leads to peritoneal infection[8]. The author of this study had 7 patients with peritonitis, 4 had inflamed diverticulum and 1 had a leak from small rupture. The appendix had features of swelling, redness in 1 patient out of all in the study.[8, 17]

A rare presentation of Meckel's diverticulum with painless rectal bleeding was noted in an adult.[12] Another heterotopic pancreatic mucosa found in a report in China.[14] Instant rupture of Meckel's diverticulum was seen in 6 patients operated for acute appendicitis for which re explored.[13] Foreign body as melon seeds obstructing Meckel's diverticulum found in a patient of Turkey.[15]

Surgical options for management are considered in severe cases with manifestations of other disorders.[5] Excision of dead and diseased diverticulum laparoscopically or through laparotomy is needed. Sometimes gut removal along with malignancy is required.

Conclusion

Meckel's Diverticulum is difficult to diagnose at first place. A patient who presents with gut obstruction at late 40s may be suspected to have malignancy until proved. Sometimes strong clue may be obtained through radiological investigations. However, we have to takeMeckels as differential diagnosis on strong clinical suspicion. Rare clinical features should be kept in mind according to presentations.

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