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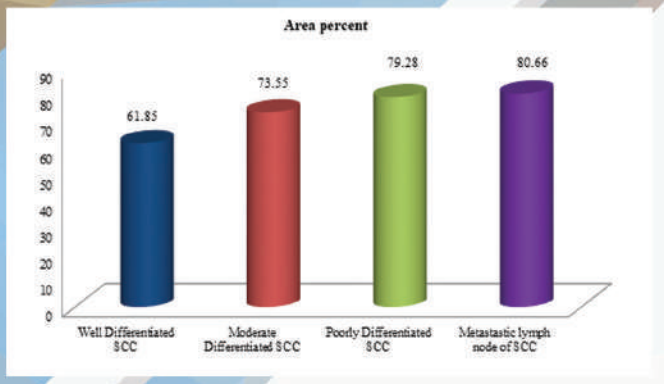

الإصدار الرابع

العدد الثاني

شتاء2021

مجلة العلوم الطبية تصدر عن الكليات الطبية بجامعة مصراتة الإصدار الرابع العدد الثاني شتاء2021

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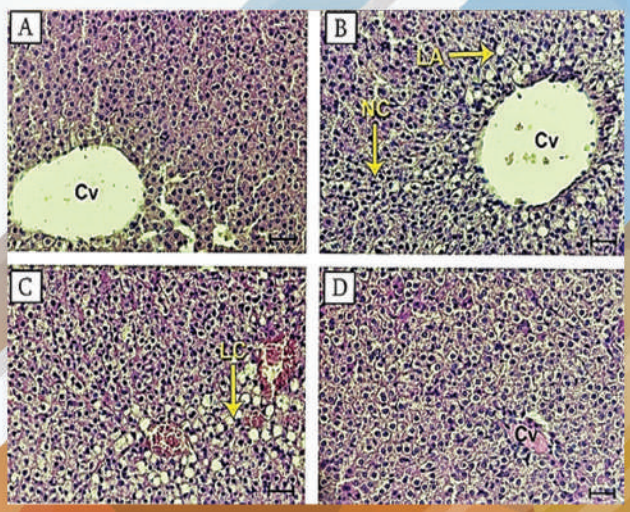

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Contents

PROTECTIVE EFFECT OF POMEGRANATE PEEL EXTRACT ON DIETARY-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

Bashir M Ayad[1], Mohamed A Elshawesh[2], Omer K Alatresh[2], Abdalla R Elgenaidi[3*]

1- Department of Physiology, Faculty of Medicine, Misurata University, Misurata, Libya.
2- Department of Medical Laboratory, Faculty of Medical Technology. Misurata, Libya.
3- Department of Therapeutical Nutrition, Faculty of Nursing and Health Sciences, Misurata University.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders that is generally associated with abnormal liver function test results. Pomegranate has been described as the nature's power fruit, and shown to have various health benefits. This study aims to explore the role of aqueous pomegranate peel extract (PPE) in attenuating NAFLD induced by high fat diet (HFD). 24 male guinea pigs were allocated into three groups (eight per group). Control (CON) group received normal chow diet. High fat diet (HFD) group, consumed HFD over six weeks to induce NAFLD. HFD-PPE group received HFD for six weeks followed by four weeks administration of PPE tea along with HFD. All animals survived until termination of the experiment, and were ultimately sacrificed at scheduled time (six weeks for the CON and HFD groups and ten weeks for the HFD-PPE group). NAFLD was evaluated histologically and by measuring serum levels of liver enzymes. There was a significant increase in the serum levels of cholesterol ($p=0.01$), triglyceride ($p=0.01$), alanine transaminase [ALT] ($p=0.01$) and aspartate transaminase [AST] ($p=0.001$) in the HFD group compared to the control. These levels were significantly lower in the HFD-PPE group compared to the HFD animals ($p=0.05$, 0.01, 0.01 and 0.001) for serum concentrations of cholesterol, triglyceride, ALT and AST respectively. A clear reduction in the extent of intrahepatocytic fat deposition was observed in the HFD-PPE animals as compared to the HFD group. This study demonstrates the potential role of PPE in the alleviation of HFD-induced NAFLD. While further studies are required to clarify the underlying mechanisms, these findings may provide the foundation for further nutritional and therapeutic developments.

KEYWORDS: HFD, NAFLD, Pomegranate, Liver enzymes.

INTRODUCTION

During the last few decades, the prevalence of overweight and obesity has increased substantially throughout the world. Overweight and obesity have been identified as key risk factors for a wide range of chronic diseases including Non-alcoholic fatty liver disease [NAFLD] [(1)]. NAFLD is asymptomatic disease that is characterised by excessive fat accumulation in hepatocytes and commonly associated with abnormal liver function tests [(2)]. NAFLD is considered to be one of the most common liver disorders in the world [(3)]. In the United States, for instance, it is estimated that about 30% of adults are affected by NAFLD, while the rate of incidence raises to over 70% in morbidly obese individuals [(4)]. Unfortunately, there is still a lack of accurate statistics upon which to make reliable estimates of the prevalence of NAFLD in Libya. Although there are no definite therapeutic drugs for NAFLD approved by regulatory agencies, some pharmaceutical agents have been recommended, e.g. pioglitazone and vitamin E, but these are not exempt from detrimental effects [(5, 6)]. Recently, there has been an emphasis on the use of herbal remedies with many people around the world resorting to phytonutrients or nutraceuticals for treatment of numerous health challenges in various national healthcare settings [(7)].

Pomegranate (*Punica granatum*) is one of the oldest edible fruits that has been known since the ancient times in the Middle East and the Mediterranean [(8)]. Pomegranate trees are long-lived, drought tolerant, and can adapt to adverse ecological conditions. Therefore, they are widely planted in various arid and semi-arid regions of the world. Anatomically, the pomegranate tree can be divided into different components, including: root, bark, leaf, flower, as well as peel and seed [(9)].

The pomegranate has been honored by being mentioned twice in the holy Quran, and designated as an example of the fruits that grow in the gardens of paradise [(9)]. Owing to its numerous beneficial effects, the pomegranate has recently been described as the nature's power fruit [(10)] that has been extensively used as a medical functional food [(11)]. Various studies have investigated the role of pomegranate fruits and extracts as possible therapeutic agents, and so far, they have shown a remarkable potential in promoting several health properties. For instance, pomegranate juice supplementation to atherosclerotic mice resulted in a significant reduction in the size of the atherosclerotic lesion as well as the number of foam cells, as compared to the control group [(12)]. Similarly, long-term consumption of pomegranate juice has also been shown to provide a great protection against cardiotoxicity and restore cardiac properties back to normal status [(13)]. In patients with carotid artery stenosis, daily intake of pomegranate juice for three years resulted in a remarkable improvement in the blood pressure, low-density

Correspondence and reprint request: Bashir M Ayad. Department of Physiology, Faculty of Medicine, Misurata University, Misurata, Libya. E-mail: ayadbm74@gmail.com

lipoprotein oxidation as well as the thickness of common carotid intima-media [14].

About 50% of the total weight of pomegranate fruits corresponds to their peel [15]. Pomegranate peel is a primary byproduct which, if not reprocessed properly, may cause essential sanitary problems, and become a possible source of environmental pollution. Several studies have recently been carried out to assist in the recycling of this refuse into a wide range of valuable products [16, 17]. Compared to the pomegranate pulp, the peel seems to have more potential health promoting effects which are apparently attributable to its higher antioxidant capacity [15]. Interestingly, among various components (e.g. peel, pulp, and seed) of 28 types of commonly consumed fruits in China, the pomegranate peel has been reported to have the most powerful antioxidant and anti-inflammatory activities [18].

Although many experimental studies have demonstrated the health beneficial effects of different pomegranate extracts, only few are available on the association between pomegranate peel and liver functions, and the results seem to be contradictory [10, 19]. Indeed, the efficacy of the medicinal plant is mainly determined by its chemical composition, which has been reported to be influenced by a variety of factors including environmental and agronomic conditions, harvest time, geographical variations as well as method of extraction [20, 21]. With this in mind, we aim to explore the potential role of pomegranate peel aqueous extract (PPE) in attenuating NAFLD induced by high fat diet (HFD) in guinea pigs.

MATERIALS AND METHODS

Twenty four healthy adult male guinea pigs (*Cavia porcellus*), aged 6-8 weeks old were used in this *in vivo* study. The study protocol was approved by the Ethics Committee of the Faculty of Medical Technology, Misurata, Libya. The animals were purchased from a commercial breeder (Misurata, Libya) and housed in stainless steel cages (eight animals per cage) in the animal house (Department of Medical Laboratory, Faculty of Medical Technology, Misurata, Libya), under standard conditions of 12 hours light-dark cycle at room temperature. Cages were provided with tap water bottles and feeders, furnished with appropriate bedding material and cleaned on a daily basis.

Preparation of pomegranate aqueous peel extract:

The fresh fruits of pomegranate, free of visible marks or defects were collected from a local farm (Misurata, Libya). Pomegranate fruits were washed, the peels were removed manually and cut into small pieces. The cut pieces were left to air dry for 15 days at room temperature, and then grained to fine powder by a commercial mill. The aqueous extract of pomegranate peel was prepared daily by adding 20g of dry powder to 1000 mL of boiled distilled water. The extract was filtered to remove the residual peel particles and left to cool down slowly to room temperature. The freshly prepared extract was orally administrated to the experimental animals at a dose level equivalent to 800mg/kg.

Experimental design:

After a week of acclimatization, during which all the experimental animals had free access to drinking tap water and standard chow, the animals were randomly assigned to three groups, each comprising eight animals: first: Lean control (CON) group, which received a normal chow diet and tap water ad libitum for six weeks. Second: High fat diet (HFD) group, which had free access to tap water and HFD for six weeks to induce NAFLD. Third: HFD-PPE group, which had unrestricted access to HFD for six weeks followed by four weeks administration of PPE tea along with HFD. All animals survived until termination of the experiment, and were ultimately sacrificed at scheduled time (six weeks for the CON and HFD groups and ten weeks for the HFD-PPE group). Blood samples (about 5 mL) as well as liver tissue were harvested. All precautions were taken to reduce the potential of pain and the number of animals included in the study.

Analyses of lipid profile and liver function markers:

Serum total cholesterol, triglyceride, as well as alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured by means of COBAS INTEGRA® 400 plus Analyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland) using commercially available kit (32 COBAS c packs) according to the manufacturer's instructions.

Histopathological examination:

The animal's livers were carefully harvested and fixed with 10 % formalin solution at room temperature for 24 hours. Fixed liver tissue specimens were dehydrated in ascending concentrations of ethanol. Thereafter, dried tissues were embedded in paraffin wax, and then a rotary microtome (pfm 3004 M medical ag, Wankelstraße 60, 50996 Köln, Germany) was used to cut tissue slices uniformly at a thickness of 5 µm. The paraffin section was transferred onto a slide and subsequently stained with haematoxylin and eosin (H&E) staining. Microscopic examination and imaging of the stained sections was carried out using Eclipse Ci-L digital microscope camera (Nikon Instruments Inc, Melville, Tokyo, Japan).

Statistical analysis:

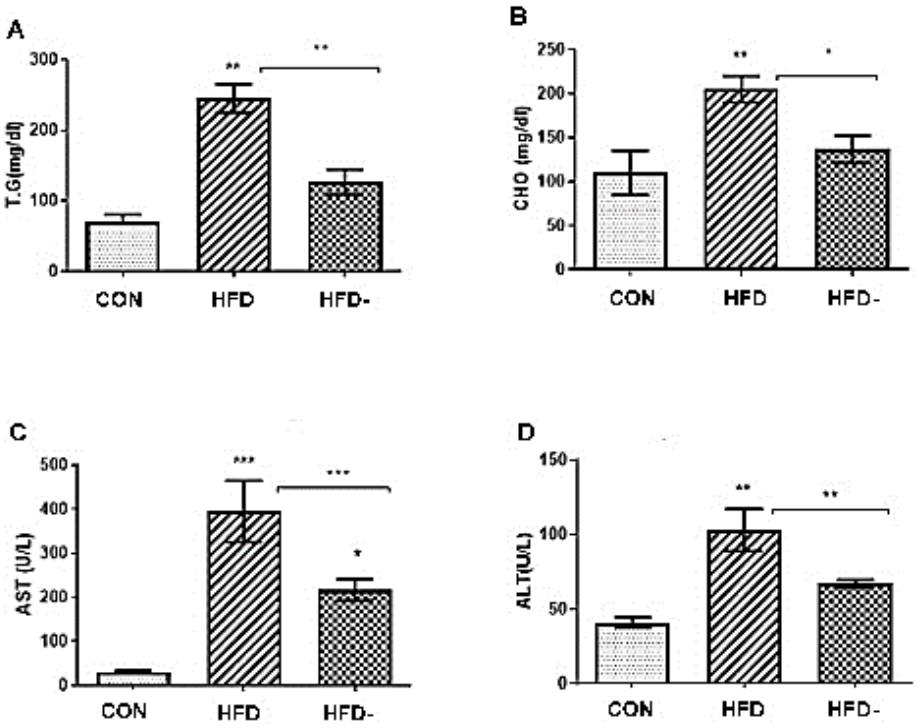
Statistical comparisons between the three experimental groups (CON, HFD, and HFD-PPE groups) were performed using one-way analysis of variance [ANOVA] (GraphPad™, version 6, Software, San Diego, CA, USA). Data were presented as Mean ± SD. Statistical significance was set at $p < 0.05$.

RESULTS

Biochemical findings:

As shown in (figure 1) (A – D), there was a significant increase in the serum levels of cholesterol (205±15 vs.

110±25 mg/dl, p=0.01), triglyceride (245±20 vs. 70±11 mg/dl, p=0.01) as well as ALT (103±14.2 vs. 41±3.4 U/L, p=0.01) and AST (395±70 vs. 31±2.5 U/L, p=0.001) in the HFD group compared to the control group. However, these levels were significantly lower in the HFD-PPE group compared to the HFD animals (137±15.5 vs. 205±15 mg/dl, p=0.05), (127±17.5 vs. 245±20 mg/dl, p=0.01), (67±2.5 vs. 103±14.2 U/L, p=0.01), (217±24 vs. 395±70 U/L, p=0.001) for serum concentrations of cholesterol, triglyceride, ALT and AST respectively.



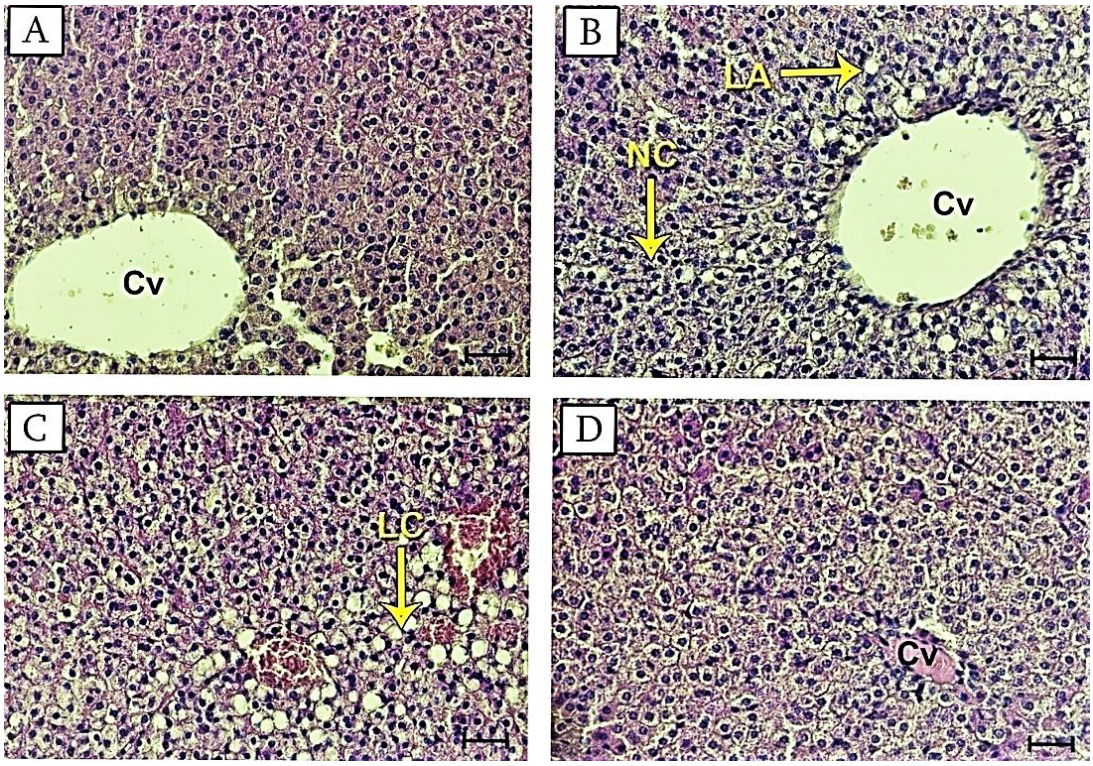
(Figure 1) Levels of biochemical findings in sera of the experimental groups.
A: Total Cholesterol; **B:** Triglyceride; **C:** Aspartate Transaminase (AST); **D:** Alanine Transaminase (ALT).
CON = control; HFD = High fat diet; PPE = pomegranate peel extract. * (p>0.05); ** (p=0.01); *** (p=0.001).

Histological findings:

Fatty liver was assessed histologically in the different animal groups using H&E staining. As visualized by light microscope, the control group of animals (Figure 2, A) showed typical lobular structure with normal hepatocytes and regular cords radiating from central veins. Compared to the control group, the HFD animals (Figure 2. B and C) displayed severe macrovesicular hepatic stenosis, revealed by accumulation of abnormal amounts of fat in a considerable fraction of hepatocytes. The intrahepatocytic fat droplets mostly appeared as one large vacuole that occupies the greater part of intracellular space, displacing the nucleus toward the periphery against the plasma membrane, leaving a clear halo of cytoplasm. Four weeks administration of PPE, (Figure 2, D), resulted in a clear recovery of the hepatic architecture, supported by a remarkable reduction in the severity of macrovesicular steanosis, in the HFD-PPE animals.

DISCUSSION

The health promoting effects of pomegranate fruits have attracted the attention of many researchers from all around the world. However, there is scarcity of studies available that have directly investigated the effect of the PPE on the NAFLD. The aim of the present study was to investigate the ameliorative effect of the aqueous PPE on NAFLD induced by HFD, using guinea pigs. Herein, HFD feeding resulted successfully in a considerable increase in the animal's serum concentrations of total cholesterol and triglyceride. Furthermore, the abnormal deposition of fat within the hepatocytes, along with the evident deterioration in liver function, clearly indicate the development of NAFLD in the HFD group of animals. However, after four weeks of PPE consumption, the treated animals showed a remarkable decline in the serum lipid profiles, together with an efficient alleviation of liver dysfunction, as indicated by the improvement in the histological features and serum levels of liver enzymes.



(Figure 2) H&E staining showing liver histopathology of A: control; B and C: HFD group; D: HFD-PPE group. Cv, central vein; LA, lipid accumulation; NC, necrotic cells; LC, Lymphocytic cell; Bar = 20 µm.400X.

In support of our findings, administration of HFD simultaneously with PPE for eight weeks could provide a useful means to improve liver function via reducing ALT and AST serum levels, as well as abolishing hepatic damage and hyperlipidaemia [11]. Likewise, the hepatoprotective effect of PPE has also been demonstrated in rat liver injury triggered by carbon tetrachloride, which is an efficient toxin used to induce liver pathological lesions that closely resemble hepatic features of NAFLD [22]. Moreover, the consumption of cupcakes fortified with PPE resulted in a substantial decline in body weight, as well as in serum levels of lipid profiles and hepatic enzymes. Accordingly, PPE could apparently be considered as a natural agent that protects against obesity, hyperlipidaemia and liver damage [23].

In the above cited studies, evaluation of the onset and progression of HFD-induced NAFLD was preferentially conducted using rat models, including albino wistar rats [10] and Sprague-Dawley rats [11, 23]. However, it is worth mentioning that rats are significantly different from humans, especially in terms of gene expression as well as activity levels of certain genes associated with hepatic lipid metabolism. Consequently, the disease pathogenesis in these rodents may not truly mimic that of human situation [24]. The use of guinea pigs as a realistic model for the demonstration of diet-induced fatty liver has strongly been recommended. Indeed, the striking similarities between these animals and humans, particularly in terms of hepatic lipoprotein mechanism and enzyme activities, further strengthens the prospect of being a more reliable model for exploring NAFLD aetiology and potential interventions [24, 25, 26].

The hyperlipidemia observed in the HFD group can unsurprisingly be attributable to the imbalance between high energy intake and energy expenditure [27]. Palatable food is linked to activation of the brain's dopamine reward system implicated in food craving behavior [28]. Furthermore, leptin is an adipose-derived hormone that is assumed to play a part in the development of obesity and NAFLD, probably via decreasing food intake and promoting energy expenditure. NAFLD has been shown to be associated with elevated leptin levels, suggesting the potential of leptin resistance [29]. Although the current study did not measure serum levels of leptin, it appears that it contributes to the anti-obesity and hepatoprotective properties of PPE throughout improving leptin sensitivity in the treated animals. In support of this, four weeks supplementation with pomegranate juice was associated with a remarkable reduction in serum leptin levels in rats susceptible to HFD-induced obesity [30]. This perspective may suggest an interesting avenue for further research.

When overproduction of reactive oxygen species (ROS) is accompanied by insufficient antioxidant defence, a state of oxidative stress arises leading to excessive cellular damage [31]. Oxidative stress has strongly been suggested to play a vital role in the onset and progression of NAFLD. In this regard, patients with stenosis and metabolic syndrome exhibited higher lipid peroxides as well as lower vitamin C and alpha-tocopherol serum levels compared to their normal peers [32]. Furthermore, the extent of oxidative

stress, as evaluated by measuring nitrite, superoxide dismutase, and catalase activities, has been shown to differ significantly based on the disease severity and histological variabilities [33]. In NAFLD, the hepatic accumulation of triglycerides is primarily due to increased rate of free fatty acid (FFA) fluxes to the liver [34]. An overload of FFAs into the hepatocytes triggers the synthesis of highly reactive free radicals via both over-reduction of the mitochondrial electron transport chain and peroxisomal oxidation [35]. In this study, for weeks administration of PPE to animals with NAFLD resulted in a substantial improvement in liver function. Although the exact mechanism by which PPE offers hepatoprotection is unknown, there is growing evidence to suggest that pomegranate peel possess stronger antioxidant properties than most other bioactive constituents of the human diet [18]. The potent antioxidant activity of the pomegranate peel is attributable to it high contents of phenolic and flavonoid compounds, which are known to have powerful scavenging capacities for ROS [36, 37]. For greater understanding of the underlying mechanisms, future studies should include assessment of additional lipid profile and oxidative stress biomarkers.

In conclusion, the present study clearly demonstrates the potential hypolipidemic, and hepatoprotective effects of the aqueous PPE. While further studies are required to shed more light on the underlying molecular mechanisms, these findings may provide the foundation for further nutritional and therapeutic developments.

REFERENCES

1- Marchesini G, Babini M. Nonalcoholic fatty liver disease and the metabolic syndrome. Minerva cardioangiologica. 2006; 54(2):229-39.

2- Clark JM, Brancati FL, Diehl AM. The prevalence and etiology of elevated aminotransferase levels in the United States. The American journal of gastroenterology. 2003; 98(5):960-7.

3- Gottlieb A, Canbay A. Why bile acids are so important in non-alcoholic fatty liver disease (NAFLD) progression. Cells. 2019; 8(11):1358.

4- Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004; 40(6):1387-95.

5- Francque S, Vonghia L. Pharmacological treatment for non-alcoholic fatty liver disease. Advances in Therapy. 2019; 36(5):1052-74.

6- Moctezuma-Velázquez C. Current treatment for non-alcoholic fatty liver disease. Revista de Gastroenterología de México (English Edition). 2018; 83(2), 125-33.

7- WHO. *WHO Guidelines on Safety Monitoring of Herbal Medicines in Pharmacovigilance Systems*. Geneva, Switzerland: World Health Organization. 2004.

8- Chandra R, Babu KD, Jadhav VT, Jaime A, Silva TD. Origin, history and domestication of pomegranate. Fruit, Vegetable and Cereal Science and Biotechnology. 2010; 1-6.

9- Rahimi HR, Arastoo M, Ostad SN. A comprehensive review of Punica granatum (pomegranate) properties in toxicological, pharmacological, cellular and molecular biology researches. Iranian journal of pharmaceutical research: IJPR. 2012; 11(2):385.

10- Moneim AE, Dkhil MA, Al-Quraishy S. Studies on the effect of pomegranate (Punica granatum) juice and peel on liver and kidney in adult male rats. Journal of Medicinal Plants Research. 2011; 5(20):5083-8.

11- Al-Shaaibi SN, Waly MI, Al-Subhi L, Tageldin MH, Al-Balushi NM, Rahman MS. Ameliorative effects of pomegranate peel extract against dietary-induced nonalcoholic fatty liver in rats. Preventive nutrition and food science. 2016; 21(1):14.

12- Aviram M, Dornfeld L, Rosenblat M, Volkova N, Kaplan M, Coleman R, Hayek T, Presser D, Fuhrman B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E–deficient mice. The American journal of clinical nutrition. 2000; 71(5):1062-76.

13- El-Wakf AM, El-Habibi ES, Barakat NM, Attia AM, Hussein AM, Ali II. Cardiovascular toxic effects of chlorpyrifos: a possible protective role for pomegranate extracts. J Clin Toxicol. 2018; 8(374):2161-0495.

14- Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A, Dornfeld L, Volkova N, Presser D, Attias J, Liker H, Hayek T. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Clinical Nutrition. 2004; 23(3):423-33.

15- Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food chemistry. 2006; 96(2):254-60.

16- Sharifi A, Chaji M. Effects of processed recycled poultry bedding with tannins extracted from pomegranate peel on the nutrient digestibility and growth performance of lambs. South African Journal of Animal Science. 2019; 49(2):290-300.

17- Talekar S, Patti AF, Vijayraghavan R, Arora A. Recyclable enzymatic recovery of pectin and punicalagin rich phenolics from waste pomegranate peels using magnetic nanobiocatalyst. Food Hydrocolloids. 2019; 89:468-80.

18- Guo C, Yang J, Wei J, Li Y, Xu J, Jiang Y. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutrition research. 2003; 23(12):1719-26.

19- Sadia H, Akter QS, Afroz R, Siddika T. Effect of Punica Granatum (Pomegranate) on serum ALT and AST in Carbon tetrachloride induced liver damage in Wistar Albino Rats. Journal of Bangladesh Society of Physiologist. 2016; 11(1):23-8.

20- Jayanthy A, Prakash KU, Remashree AB. Seasonal and geographical variations in cellular characters and chemical contents in Desmodium gangeticum (L.) DC.–an ayurvedic medicinal plant. Int J Herbal Med 2013; 1: 34.-7.
21- Liu Y, Chen P, Zhou M, Wang T, Fang S, Shang X, Fu X. Geographic variation in the chemical composition and antioxidant properties of phenolic compounds from Cyclocarya paliurus (Batal) Iljinskaja leaves. Molecules. 2018; 23(10):2440.
22- Chheda TK, Shivakumar P, Sadasivan SK, Chanderasekharan H, Moolemath Y, Oommen AM, Madanahalli JR, Marikunte VV. Fast food diet with CCl4 micro-dose induced hepatic-fibrosis–a novel animal model. BMC gastroenterology. 2014; 14(1):89.
23- Lamiaa ML, Eman SA. The Impact of Pomegranate Peel-fortified Cupcakes on Weight Loss. International Journal of Pharmaceutical Research & Allied Sciences. 2019; 8(3).
24- DeOgburn R, Murillo AG, Fernandez ML. Guinea pigs as models for investigating non-alcoholic fatty liver disease. Integr. Food, Nutr. Metab. 2016; 3:309-13.
25- Fernandez ML, Volek JS. Guinea pigs: a suitable animal model to study lipoprotein metabolism, atherosclerosis and inflammation. Nutrition & metabolism. 2006; 3(1):17.
26- Ye P, Cheah IK, Halliwell B. High fat diets and pathology in the guinea pig. Atherosclerosis or liver damage?. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2013; 1832(2):355-64.
27- Romieu I, Dossus L, Barquera S, Blottière HM, Franks PW, Gunter M, Hwalla N, Hursting SD, Leitzmann M, Margetts B, Nishida C. Energy balance and obesity: what are the main drivers?. Cancer Causes & Control. 2017; 28(3):247-58.
28- Sorokowska A, Schoen K, Hummel C, Han P, Warr J, Hummel T. Food-related odors activate dopaminergic brain areas. Frontiers in Human Neuroscience. 2017; 11:625.
29- Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Liddle C, Samarasinghe D, George J. Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity?. Hepatology. 2002; 36(2):403-9.
30- Ahmed MM, Samir ES, El-Shehawi AM, Alkafafy ME. Anti-obesity effects of Taif and Egyptian pomegranates: Molecular study. Bioscience, biotechnology, and biochemistry. 2015; 79(4):598-609.
31- Ayad BM, Van der Horst G, Du Plessis SS. Revisiting the relationship between the ejaculatory abstinence period and semen characteristics. International journal of fertility & sterility. 2018; 11(4):238-46.
32- Palmieri VO, Grattagliano I, Portincasa P, Palasciano G. Systemic oxidative alterations are associated with visceral adiposity and liver steatosis in patients with metabolic syndrome. The Journal of nutrition. 2006; 136(12):3022-6.
33- Stiuso P, Scognamiglio I, Murolo M, Ferranti P, De Simone C, Rizzo MR, Tuccillo C, Caraglia M, Loguercio C, Federico A. Serum oxidative stress markers and lipidomic profile to detect NASH patients responsive to an antioxidant treatment: a pilot study. Oxidative medicine and cellular longevity. 2014; 169216.
34- Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology. 2010; 51(2):679-89.
35- Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radical Biology and Medicine. 2012; 52(1):59-69.
36- Kandylis P, Kokkinomagoulos E. Food applications and potential health benefits of pomegranate and its derivatives. Foods. 2020; 9(2):122.
37- Smaoui S, Hlima HB, Mtibaa AC, Fourati M, Sellem I, Elhadef K, Ennouri K, Mellouli L. Pomegranate peel as phenolic compounds source: Advanced analytical strategies and practical use in meat products. Meat science. 2019; 158:107914.

EXPRESSION OF EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER (EMMPRIN) AS A BIOMARKER IN ORAL SQUAMOUS CELL CARCINOMA (OSCC)

Ben amer A.M[1], Omer E M[2], Elsheikh S.M[3]

1- Demonstrator, Department of Oral Pathology, Faculty of Dentistry, misurata University.
2- Lecutuer of oral pathology, Faculty of Dentistry, Alexandria University Egypt.
3- Professor, Department of Oral Pathology, Faculty of Dentistry, Alexandria University Egypt.

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ABSTRACT

EMMPRIN is a widely distributed cell surface glycoprotein that belongs to immunoglobulin (Ig) super family. It is inducing the production of extracellular matrix metalloproteinase (MMP) enzymes and play important role in angiogenesis via stimulation of vascular endothelial growth factor (VEGF). The aim of the present work is to evaluate and asses the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) in oral squamous cell carcinomas and to compare the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) in different types of oral squamous cell carcinomas. Thirty diagnosed cases of Oral squamous cell carcinoma (OSCC) were selected and immunohistochemistry was performed for EMMPRIN. All cases showed positive EMMPRIN expression with different intensity. This study concluded that the elevated expression of EMMPRIN levels correlate with tumor proliferation, angiogenesis, metastasis and invasion.

KEY WORDS: Oral squamous cell carcinoma (OSCC), EMMPRIN.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide with an estimated 211,500 new cases per year (2.6% of all cancers) and over 120,000 deaths every year[(1)]. The etiology of oral cancer is multi-factorial, with multiple carcinogenic agents affecting the oral mucosa[(2)].

EMMPRIN is a widely distributed cell surface glycoprotein that belongs to immunoglobulin (Ig) super family[(3)]. It is expressed in numerous cells; including platelets, fibroblasts, T-lymphocytes and especially in cancer cells[(4-7)].

The major function of EMMPRIN has been implicated in many biological functions, such as; in the developing nervous system[(8)], embryo implantation, spermatogenesis[(9)] retinal development[(10)], and in immune cell activation[(11)]. Elevated EMMPRIN expression levels correlate with tumor proliferation, angiogenesis, metastasis and invasion[(12,13)]. It was found that over expression of EMMPRIN / CD147 in cancer tissues is associated with poor prognosis of patients with several types of solid tumors[(14)].

The number of studies focusing on EMMPRIN expression in oral squamous cell carcinoma (OSCC) is limited, so the aim of the present study is to detect the expression of EMMPRIN immunohistochemically in different oral squamous cell carcinoma (OSCC) and to compare between them**.**

MATERIALS AND METHODS

Biopsies:

Thirty patients clinically diagnosed with OSCC were selected from the Cranio-Maxillofacial and Plastic Surgery Department at the Faculty of Dentistry, Alexandria University. a written informed consent was taken from all the patients and 10 patients agreed to be photographed before surgery. Biopsies were taken from the tumor tissue and fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax using the conventional procedures. Serial sections of 3-4 µm thick were placed on glass slides and stained using hematoxylin and eosin (H&E) for routine histopathological examination.

Immunohistochemical staining of EMMPRIN:

Immunohistochemical marker of primary rabbit polyclonal antibody EMMPRIN Glut 1 Cat. # E2260-03 (0.5 ml), (US Biological life science) was used. Strept-Avidin Biotin-peroxiadase complex method (LSAB) was used. Serial sections 4-5 µm thick were taken from the previously used for H&E blocks. The slide will be mounted on poly-L-lysine coated glass slides. Two sections will be obtained for the positive test slides and third one for the negative control by omitting the primary antibody. The tissue sections were deparaffinized in xylene, rehydrated in graded ethanol and incubated in 0.3% hydrogen peroxide solution to block the endogenous peroxidase. The specimens were washed with an appropriately characterized, diluted and were incubated with the primary antibody of EMMPRIN. Exposure to biotinylated link antibody and labeled streptavidin-biotin-peroxidase complex was done to bind the primary antibody. Staining was completed by incubation in substrate-chromogen solution and hematoxylin counter stain. Immuno-expression of EMMPRIN will be evaluated by using image analyzer to evaluate both mean area percent and mean optical density.

The results were recorded and statistically analyzed using (ANOVA) test.

RESULTS

In the present work, a total of 30 patients with OSCC were included. The patients' age ranged between 30 and 76 with a mean of (58.1years). Twenty patients (66.6%) were males and ten patients (33.3%) were females. As regards to location, the most common site of occurrence was the alveolar ridge, 15cases (50%), followed by the lateral side of the tongue 10 cases (33.3%) and the buccal mucosa 5 cases (16.6%).

Clinical data regarding the site of the lesion is presented in the **(table 1)**.

(Table 1) Clinical Data of Squamous Cell Carcinoma Cases According to the Location.

Diagnosis	Age (y)/sex	Location	Presence of Lymph Node Metastatic Deposits
Poorly differentiated squamous cell carcinoma	30/F	Alveolar ridge	x
Well differentiated squamous cell carcinoma	75/M	Alveolar ridge	√
Poorly differentiated squamous cell carcinoma	60/M	Alveolar ridge	x
Moderate differentiated squamous cell carcinoma	72/M	Alveolar ridge	x
Moderate differentiated squamous cell carcinoma	70/M	Alveolar ridge	x
Well differentiated squamous cell carcinoma	73/M	Alveolar ridge	x
Well differentiated squamous cell carcinoma	63/F	Tongue	x
Poorly differentiated squamous cell carcinoma	64//F	Tongue	x
Moderate differentiated squamous cell carcinoma	33/F	Tongue	x
Well differentiated squamous cell carcinoma	51/M	Tongue	x
Moderate differentiated squamous cell carcinoma	35/F	Tongue	x
Poorly differentiated squamous cell carcinoma	76/M	Buccal mucosa	x
Moderate differentiated Squamous cell carcinoma	50/F	Buccal mucosa	√
Moderate differentiated squamous cell carcinoma	59/M	Buccal mucosa	x
Well differentiated squamous cell carcinoma	61/F	Palatal mucosa	x

Immunohistochemical Results:

In the present immunohistochemical study, routinely formalin fixed, paraffin-embedded 30 patients with OSCC biopsies were used. This was done to detect the extracellular matrix metalloproteinase inducer (EMMPRIN) expression along with the normal control.

The intensity of immunostaining of EMMPRIN was calculated in terms of mean area percent and mean optical density by the computer image analyzer.

Pattern of EMMPRIN Immunostaining in Normal Control Sections:

All biopsies of normal oral mucosa (no=2) Showed positive immunosignals for EMMPRIN which is limited in the basal cell layer.

Pattern of EMMPRIN Immunostaining in Squamous Cell Carcinomas:

EMMPRIN expression was analyzed in 30 squamous cell carcinoma biopsies. They all showed positive expression.

Well differentiated SCC (n=10) showed diffuse positive cytoplasmic immunosignals of EMMPRIN in the malignant epithelial cells forming the keratin pearls, while the nuclei were free from any reaction (fig.a) *Moderately differentiated SCC* (n=8) showed positive cytoplasmic immunoreaction of EMMPRIN. The anaplastic cells formed epithelial nest, which demonstrated membranous, sometimes perinuclear immunosignals, the nuclei were free from any reaction (fig.b) *In the poorly differentiated SCC cases* (n=7) the intense cytoplasmic EMMPRIN immunopositivity was detected in the highly anaplastic malignant epithelial cells. It showed different abnormal mitotic figures (fig.c) *The metastatic lymph nodes* (n=5) of SCC revealed strong positive diffuse immunosignals of EMMPRIN within keratin pearls (fig.d).

Correlating EMMPRIN Immunoexpression in Different Grades of Squamous Cell Carcinoma:

Comparing different grades of squamous cell carcinomas (SCC) and metastatic lymph node of squamous cell carcinoma according to the area percent of EMMPRIN immunoexpression was done. The greatest mean value was in metastatic lymph node of squamous cell carcinoma (80.66±11.05) and the lowest value was in well differentiated SCC (61.85±1.678). ANOVA test revealed a statistically significant difference (p=0.003). Tukey's post hoc test revealed no significant difference between moderately or poorly differentiated squamous cell carcinoma and metastatic lymph node of squamous cell carcinoma, (table 2), (figure 1).

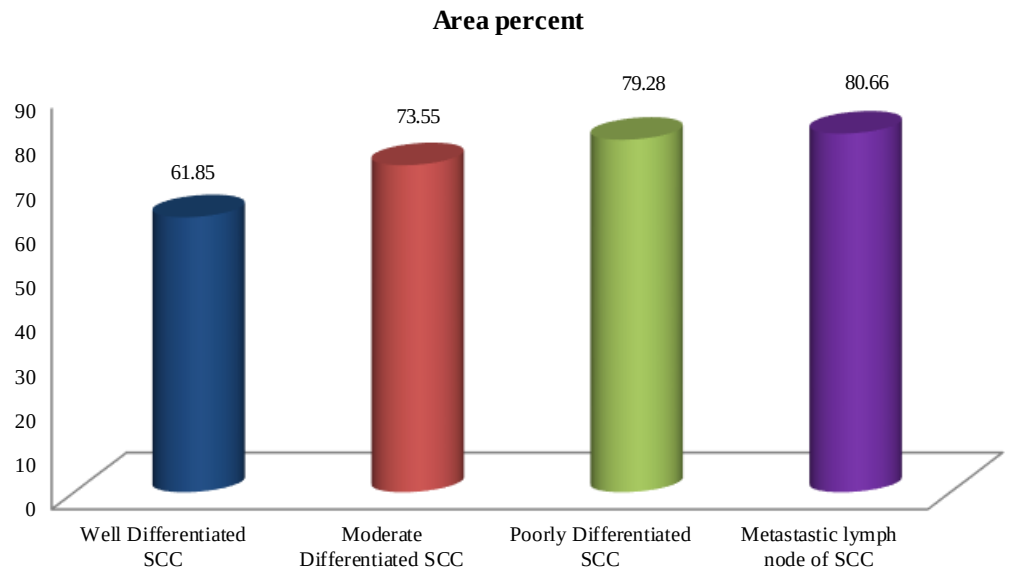
Comparing different grades of squamous cell carcinomas (SCC) and metastatic lymph node of squamous cell carcinoma according to the optical density of EMMPRIN immunoexpression was done. The greatest mean value was in metastatic lymph node of squamous cell carcinoma (83.49±7.32) and the lowest value was in well differentiated SCC (59.81±13.59). ANOVA test revealed a statistically significant difference (p=0.034). Tukey's post hoc test revealed no significant difference between moderately or poorly differentiated squamous cell carcinoma and metastatic lymph node of squamous cell carcinoma. (Table 3) and (figure 2).

(Table 2) Comparison between Different Histological Types of Squamous Cell Carcinomas (SCC) according to the Mean Area Percent of EMMPRIN immunoexpression using ANOVA Test.

	Well differentiated SCC (4 cases)	Moderate differentiated SCC (5 cases)	Poorly differentiated SCC (4 cases)	Metastatic lymph node of SCC (2 cases)
Mean ±SD	61.85a±1.68	73.55b±6.12	79.28b±3.66	80.66b±11.05
F value	8.85			
P value	0.003*			

**statistically significant*

Tukey's post hoc test: means with different superscript letters are significantly different

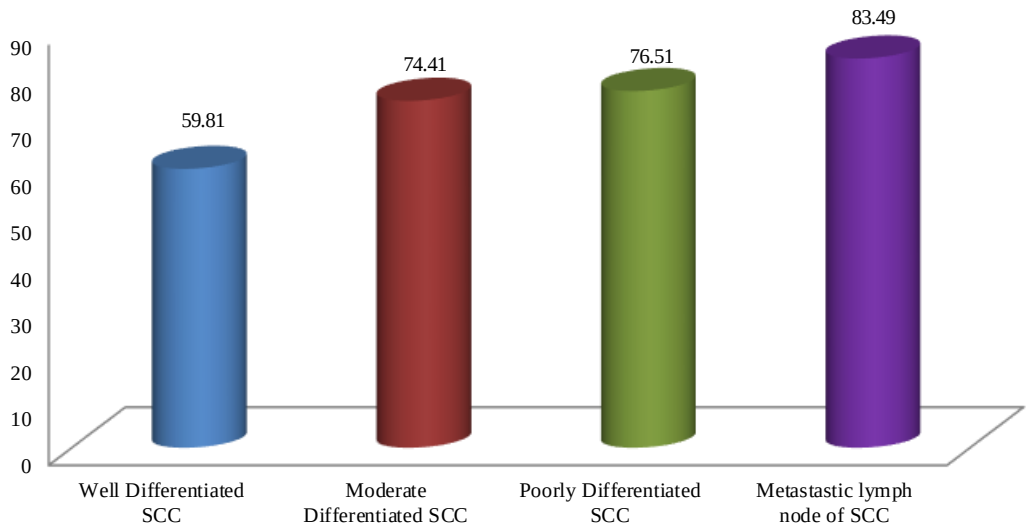


(Figure 1) Column Chart Showing the Mean Area Percent of EMMPRIN Immunoexpression in Different Histological Types of Squamous Cell Carcinomas.

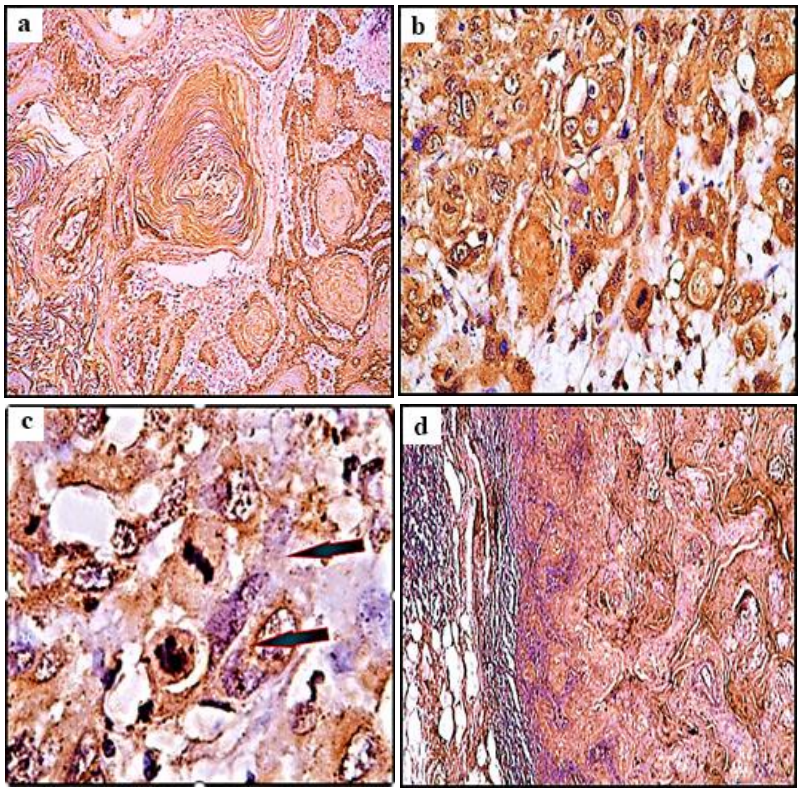
(Table 3) Comparison between Different Histological Types of Squamous Cell Carcinomas according to the Mean Optical Density of EMMPRIN Immunoexpression using ANOVA Test.

	Well differentiated SCC (4 cases)	**Moderate differentiated SCC (5 cases)**	**Poorly differentiated SCC (4 cases)**	**Metastatic lymph node of SCC (2 cases)**
Mean ±SD	59.81[a]±13.59	74.41[a, b]±5.07	76.51[a, b]±6.15	83.49[b]±7.32
F value	4.156			
P value	0.034*			

**statistically significant*
Tukey's post hoc test: means with different superscript letters are significantly different.



(Figure 2) Column Chart Showing the Mean Optical Density of EMMPRIN Immunoexpression in Different Histological Types of Squamous Cell Carcinomas.



(Figure 3)
(a) Well Differentiated Squamous Cell Carcinoma Showing Diffuse Positive Cytoplasmic Immunosignals of EMMPRIN in the Malignant Epithelial Cells Forming the Keratin Pearls and Epithelial Nests. (x100).
(b) Moderate Differentiated Squamous Cell Carcinoma Demonstrating Evident Positive Immunoreaction of EMMPRIN in All the Malignant Cell Nests. (x400).
(c) poorly Differentiated Squamous Cell Carcinoma Revealing Positive Intense Cytoplasmic Immunosignals in the Anaplastic Epithelial Cells. Note the Abnormal Mitotic Figures (Arrows). (x1000)
(d) Metastatic Lymph Node of Well Differentiated Squamous Cell Carcinoma Revealing Strong Positive Diffuse Immunosignals of EMMPRIN within Keratin Pearls (x32).

DISCUSSION

Oral cancer remains a major public health problem with almost 300,000 new cases worldwide[1,15]. New insights in cancer diagnosis and therapy have not changed significantly, during the last decades the survival rate for oral cancer is around 50%[15]. Oral tumorigenesis is a multistep process caused by accumulation of multiple genetic and epigenetic alterations. The comprehension of the molecular pathways involved in this process may originate special biological markers able to differentiate tumors with a more or less aggressive behavior. These markers may contribute to identify and stratify patients with greater precision to the most appropriate treatment plan[16].

The present study included 30 cases oral *squamous cell carcinoma*; the age range of these patients was between (30-76 years) with a mean age 58 years. It has been accepted for a long time that SCCs are associated with old age[17, 18].

In this research, the encountered squamous cell carcinoma cases showed that the alveolar ridge was the most prevalent site of occurrence, followed by the tongue and the buccal mucosa. This may be due to the poor oral hygiene of the presented cases. This is in accordance with *Effiom et al*[19].

An increasing body of evidence suggests that extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein present on the surface of tumor cells modulates key steps of the metastatic cascade. Therefore, might play a crucial role in the progression of carcinomas[20,21]. It induces angiogenesis, tumor invasiveness and multidrug resistance depending on stimulation of VEGF and MMPs production overcome "natural" barriers, such as the basement membrane and to spread locally and subsequently also reach lymphatic and blood vessels and metastasize[22]. It also elevates urokinase-type plasminogen activator (uPA) that is important in tumor progression[23].

In the present research, *control sections* included normal mucosa and squamous cell papilloma. All the examined normal mucosa specimens showed only positive cytoplasmic EMMPRIN immunosignals in the basal cell layer. This is consistent with the findings reported by, *Riethdorf et al*[7], *Siu et al*[24] and *Ayva et al*[25], *Vigneswaran et al*[26].

In the present study, EMMPRIN immunoexpression was evaluated using the computer image analyzer. All the examined *squamous cell carcinoma* cases revealed high expression of extracellular matrix metalloprotein-

ase inducer. The expression was observed in cytoplasm and membrane of the malignant epithelial cells. This is in concordance with other studies[7,27,28]. Our examined squamous cell carcinoma cases (moderate and poorly differentiated type) as well as metastatic SCC to lymph nodes showed statistically significant overexpression of EMMPRIN more frequently than the well differentiated type. This is in accordance with *Riethdorf et al.*[7] This indicates that EMMPRIN might play an important role in SCC progression and invasion.

In a recent study, conducted by *Monteiro et al*[29]. In their work on squamous cell carcinomas, they found that a positive association of EMMPRIN expression with histological grade, where moderate and poorly differentiated tumors presented EMMPRIN expression more often than well differentiated ones. They also added that this glycoprotein overexpression occurs at an early step of oral carcinogenesis and contributes to oral tumorigenesis and that this marker may serve as a reliable biological marker to identify high risk subgroups[29]. These findings agree with the findings of the present work. It also suggests that increased EMMPRIN expression could be a negative prognostic factor in SCC.

Comparing different grades of squamous cell carcinomas (SCC) and metastatic lymph node of squamous cell carcinoma according to the area percent of EMMPRIN immunoexpression was done. The greatest mean value was in metastatic lymph node of squamous cell carcinoma and the lowest value was in well differentiated SCC. Comparing different grades of squamous cell carcinomas (SCC) and metastatic lymph node of squamous cell carcinoma according to the optical density of EMMPRIN immunoexpression was done. The greatest mean value was in metastatic lymph node of squamous cell carcinoma and the lowest value was in well differentiated SCC. Moreover, well, moderately and poorly differentiated SCC didn't significantly differ from each other. Well differentiated SCC revealed a significant difference from the metastatic lymph node of squamous cell carcinoma only. This in accordance with *Zucker et al*[21], *Nabeshima et al*[30] and *Huang et al*[13] studies, they reported that EMMPRIN expression is linked to a more aggressive type of cancer. They found that over expression of EMMPRIN is a frequent and important event in head and neck cancer invasion and metastasis. They also added that major function of such glycoprotein is to stimulate the synthesis of the extracellular matrix metalloproteinase family.

CONCLUSIONS

Based on the results of the present study, expression of EMMPRIN in moderate, poorly differentiated and metastatic SCC was higher than well differentiated ones. Therefore, it might play an important role in SCC progression and invasion. Further studies with larger sample size are required to clarify the correlation between EMMPRIN expression and squamous cell carcinomas (SCC).

REFERENCES

1- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61:69-90.

2- Jones DL, Rankin KV. Oral Cancer and Associated Risk Factors. In: Bhatt AA, editor. Prevention in Clinical Oral Health Care. St Louis: Mosby Yearbook; 2008. p. 68-77.

3- Weidle UH, Scheuer W, Eggle D, Klostermann S, Stockinger H. Cancer-related issues of CD147. Cancer Genomics Proteomics 2010; 7:157-69.

4- Gabison EE, Hoang-Xuan T, Mauviel A, Menashi S. EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair. Biochimie 2005; 87:361-8.

5- Schmidt R, Bultmann A, Fischel S, Gillitzer A, Cullen P, Walch A, et al. Extracellular matrix metalloproteinase inducer (CD147) is a novel receptor on platelets, activates platelets, and augments nuclear factor kappaB-dependent inflammation in monocytes. Circ Res 2008; 102:302-9.

6- Ruiz S, Castro-Castro A, Bustelo XR. CD147 inhibits the nuclear factor of activated T-cells by impairing Vav1 and Rac1 downstream signaling. J Biol Chem 2008; 283:5554-66.

7- Riethdorf S, Reimers N, Assmann V, Kornfeld JW, Terracciano L, Sauter G, et al. High incidence of EMMPRIN expression in human tumors. Int J Cancer 2006; 119:1800-10.

8- Agrawal SM, Yong VW. The many faces of EMMPRIN - roles in neuroinflammation. Biochim Biophys Acta 2011; 1812:213-9.

9- Bi J, Li Y, Sun F, Saalbach A, Klein C, Miller DJ, et al. Basigin null mutant male mice are sterile and exhibit impaired interactions between germ cells and Sertoli cells. Dev Biol 2013; 380:145-56.

10- Hori K, Katayama N, Kachi S, Kondo M, Kadomatsu K, Usukura J, et al. Retinal dysfunction in basigin deficiency. Invest Ophthalmol Vis Sci 2000; 41:3128-33.

11- Renno T, Wilson A, Dunkel C, Coste I, Maisnier-Patin K, Benoit de Coignac A, et al. A role for CD147 in thymic development. J Immunol 2002; 168:4946-50.

12- Voigt H, Vetter-Kauczok CS, Schrama D, Hofmann UB, Becker JC, Houben R. CD147 impacts angiogenesis and metastasis formation. Cancer Invest 2009; 27:329-33.

13- Huang Z, Tan N, Guo W, Wang L, Li H, Zhang T, et al. Overexpression of EMMPRIN Isoform 2 Is Associated with Head and Neck Cancer Metastasis. PLoS One 2014; 9: e91596.

14- Li Y, Xu J, Chen L, Zhong WD, Zhang Z, Mi L, et al. HAb18G (CD147), a cancer-associated biomarker and its role in cancer detection. Histopathology 2009; 54:677-87.

15- Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009; 45:309-16.

16- Hahn WC, Weinberg RA. Rules for making human tumor cells. N Engl J Med 2002; 347:1593-603.

17- Neville BW, Day TA. Oral cancer and precancerous lesions. CA Cancer J Clin 2002; 52:195-215.

18- Pires FR, Ramos AB, Oliveira JB, Tavares AS, Luz PS, Santos TC. Oral squamous cell carcinoma: clinicopathological features from 346 cases from a single oral pathology service during an 8-year period. J Appl Oral Sci 2013; 21:460-7.

19- Effiom OA, Adeyemo WL, Omitola OG, Ajayi OF, Emmanuel MM, Gbotolorun OM. Oral squamous cell carcinoma: a clinicopathologic review of 233 cases in Lagos, Nigeria. J Oral Maxillofac Surg 2008; 66:1595-9

20- Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. Cancer Res 2001; 61:2276-81.
21- Zucker S, Hymowitz M, Rollo EE, Mann R, Conner CE, Cao J, et al. Tumorigenic potential of extracellular matrix metalloproteinase inducer. Am J Pathol 2001; 158:1921-8.
22- Van der Jagt MF, Wobbes T, Strobbe LJ, Sweep FC, Span PN. Metalloproteinases and their regulators in colorectal cancer. J Surg Oncol 2010; 101:259-69.
23- Lescaille G, Menashi S, Cavelier-Balloy B, Khayati F, Quemener C, Podgorniak MP, et al. EMMPRIN/CD147 upregulates urokinase-type plasminogen activator: implications in oral tumor progression. BMC Cancer 2012; 12:115.
24- Siu A, Chang J, Lee C, Lee S, Ramos DM. Expression of EMMPRIN modulates mediators of tumor invasion in oral squamous cell carcinoma. J Calif Dent Assoc 2013; 41:831-8.
25- Ayva SK, Karabulut AA, Akatli AN, Atasoy P, Bozdogan O. Epithelial expression of extracellular matrix metalloproteinase inducer/CD147 and matrix metalloproteinase-2 in neoplasms and precursor lesions derived from cutaneous squamous cells: An immunohistochemical study. Pathol Res Pract 2013; 209:627-34.
26- Vigneswaran N, Beckers S, Waigel S, Mensah J, Wu J, Mo J, et al. Increased EMMPRIN (CD 147) expression during oral carcinogenesis. Exp Mol Pathol 2006; 80:147-59.
27- Maria Degado, Luý ´ s Monteiro, Barbas Amaral, Sara Ricardo, Fernanda Garcês, Carlos Lopes. EMMPRIN expression in oral squamous cell carcinomas. Oral Oncology 2013;49: S4–S79.
28- Cao Z, Xiang J, Li C. Expression of extracellular matrix metalloproteinase inducer and enhancement of the production of matrix metalloproteinase-1 in tongue squamous cell carcinoma. Int J Oral Maxillofac Surg 2009; 38:880-5.
29- Monteiro LS, Delgado ML, Ricardo S, Garcez F, do Amaral B, Pacheco JJ, et al. EMMPRIN expression in oral squamous cell carcinomas: correlation with tumor proliferation and patient survival. Biomed Res Int 2014; 2014:905680.
30- Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M. Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Int 2006; 56:359-67.

PREVALENCE OF IRON DEFICIENCY ANEMIA (IDA) AMONGST NURSING STUDENTS OF MISURATA UNIVERSITY, LIBYA

Ayman Mustafa*; Ahmed Elhamroush; Abdalla Elgenaidi; Salem Elwahaishi; Fatima Ageel
Faculty of Nursing and Health Sciences, Misurata University, P.O. Box: 2478, Misurata, Libya

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ABSTRACT

Iron deficiency anemia (IDA) is the most common type of anemia. Anemia is defined as a clinical condition characterized by a reduction in hemoglobin concentration of blood or the number of red blood cells (RBCs) is poorly below the normal. The present study was conducted to investigate about prevalence of iron deficiency anemia among nursing students and elucidate the correlations between hemoglobin level, gender, physical signs, and clinical symptoms. Sixty-one students aged 18-30 years were selected randomly, during the period from April up to December 2018. Hemoglobin (Hb) level was measured for each respondent. Pearson correlation was used to find a correlation between Hb and other parameters. where $P<0.05$ was considered statistically significant. The results showed that, there was 16% found to be suffering from anemia, when 28% of females had diagnosed low Hb levels with anemia. A significant ($P<0.05$) correlation between Hb level, sex, pale-colored inside lower eyelids, general pallor and heartbeat were detected. There were no significant *($P>0.05$)* correlation between Hb level and sore mouth, anorexia, headache occurrence, dizziness and breath shortness. It was detected that, the prevalence of mild anemia among nursing students have been measured. The nutritional interventions such as change lifestyle, food fortification and diet diversification should be recommended to prevent the occurrence of anemia complications.

KEY WORDS: Anemia, Hemoglobin, Misurata, Nursing, Students.

INTRODUCTION

Iron deficiency (ID) is the most common nutritional deficiency worldwide and an important public health problem, especially in developing countries. There is no clear data about how many individuals are affected by iron deficiency worldwide, but it is estimated that ID is present in most of the pre-school children and pregnant women in developing countries and at least 30- 40% in developed countries when anemia is used as an indirect indicator of ID. WHO is defines anemia as Hb < 130 g/L in men older than 15 years, 110 g/L in pregnant women, and <120 g/L in non-pregnant women older than age 15 years[1,2].

The prevalence of anemia increases with age and in the hospital setting. Anemia decreases the capacity for work and increases health care costs. Iron deficiency is the predominant cause of anemia across countries and in both sexes, with women more commonly afflicted. Iron deficiency is also associated with restless legs syndrome (RLS), diminished quality of life, fatigue, impaired cognitive function, and infertility, all of which may occur in the absence of anemia and may be reversed with iron therapy[3,4,5,6].

ID and IDA are global health problems and common medical conditions seen in every day clinical practice. Since anemia is the most important indicator of iron deficiency, the terms ID and IDA are often used interchangeably. However, ID may develop in the absence of anemia and the tissues may be affected by this condition. Iron deficiency is manifested in different stages. If an iron requirement is below the intake, iron stores are reduced primarily. After the iron stores are reduced, hemoglobin levels may stay normal for a while which means that iron deficiency is observed in the absence of anemia. At this time, only plasma ferritin levels and plasma transferrin saturation are reduced. Negative iron balance which continues after iron stores are exhausted is manifested with decreased hemoglobin. Conclusively, a reduced body iron store has been defined as ID and the worsening of this condition and development of anemia is defined as IDA[7].

Objectives of this study were to:

- Exam iron deficiency anemia amongst students of nursing and health sciences.
- Measurement of prevalence of iron deficiency anemia among students.
- Find out the relationship between hemoglobin level versus physical signs, clinical symptoms and gender.

MATERIALS AND METHODS

Area of study:

Misurata is a city in the Misurata District in northwestern Libya, situated 187 km (116 mi) to the east of Tripoli and 825 km (513 mi) west of Benghazi on the Mediterranean coast near Cape Misurata. With a population of about 281,000, it is the third-largest city in Libya, after Tripoli and Benghazi. It is the capital city of the Misurata District and has been called the trade capital of Libya. It has lied at longitude is 32 °.377533"N and Latitude is 15°.092017"E. It located is 7 meters' height, which is equal to 23 ft. above sea level.

Research design:

This is a descriptive study that used to determine the knowledge, awareness about anemia occurrence

*Correspondence and reprint request: Ayman Mustafa. Faculty of Nursing and Health Sciences, Misurata University, P.O. Box: 2478, Misurata, Libya.
E-mail: aymanssw@hotmail.com*

among nursing students. The questionnaire was personally conducted to the student in Faculty Nursing and Health Sciences, the survey was involved information's about age, gender, blood transfusion, physical signs, and clinical symptoms, related to iron deficiency anemia.

Study population:

All of the participants were informed with study purpose, procedures, and significance. They were signed the study consent forms. The study was approved by the department board, and all procedures were followed the ethical standards of the Misurata University. The study was targeted the students of the Faculty of Nursing and Health Sciences of Misurata University. Sixty-one students aged18-30 years were selected randomly as shown in (table 1).

Eligibility was determined through the following inclusion criteria: *1*) age 18–30 years; 2) non alcoholism and nonuser of tobacco products; *3*) generally healthy; *4*) not pregnant or lactating in the past 6 month; *5*) not clinically diagnosed with an eating disorder; *6*) no metabolic, hormonal, and/or neural conditions, diseases, or medications that influence metabolism or food intake; *7*) no known bleeding disorders.

Study duration

The study was conducted within six months. From April up to end of December 2018. The duration was distributed among data collection, analysis and interpretation and report writing.

Data collection and procedures:

Questionnaires

According to a population of the study, many students from the Faculty Nursing and Health Sciences were given a personal interview and a questionnaire after that blood samples should be taken.

Blood samples collection and preparation

The 61 blood samples were obtained during the April up to end of July 2018. When only one blood sample was drawn out from each interviewed student. 2.5 ml of blood sample was drawn out into an anticoagulant tube and caped loosely. Each tubes containing a 2.5 (EDTA) Ethylene Di Amine Tetra Acetic Acid. All blood samples were collected and transferred immediately to laboratory analysis.

Blood analysis

In the laboratory, 61 of EDTA blood samples were used to analyze complete blood count (CBC). The analysis was done using the Sysmex Automated Hematology Analyzer device (XK-21N-2012) made in German. Respondents were classified according to the hemoglobin level.

Data quality management

A structured questionnaire was prepared by English and translated into Arabic language. The pre-test of the questionnaire was done before actual data collection to see for accuracy and response and to estimate which time it is needed.

Statistical analysis

To perform calculations for statistical analysis, SPSS Statistical Version 18 and Graphs were used. The descriptive statistical method was represented in the Frequency and Percent as well as Pie Chart. Pearson correlation was used to study the relationship between two variables. It is significant if P-value is less than 0.05.

RESULTS AND DISCUSSION

The prevalence of anemia is an important health indicator when it is used with other measurements of iron status, hemoglobin concentration can provide information about the severity of iron deficiency. The physical, clinical and laboratory measurements have been taken directly. The data were collected according to specified criteria of eligibility. As it is shown in (table 1). The distribution of study samples according to age groups and gender, it is indicating the majority of respondents about 45 out of 61 were attributed to age group (18-20). Whereas, other students of age groups (21-23), (24-26) and (27-30) were recorded 21%, 3%, and 2% respectively. The majority of respondents as male, they were 52% whereas, female 48% of the total of 61 samples.

(Table 1) Distribution of study sample by age and gender

Age	Frequency	Percent
18-20	45	74%
21-23	13	21%
24-26	2	3%
27-30	1	2%
Total	61	100%
Distribution of study sample by gender		
Male	32	52%
Female	29	48%

(Table 2) shows the classification of population study according to hemoglobin level. Generally, the results showed that the majority 51 students were classified normal individuals at the normal level of hemoglobin compared to 10 of students were classified abnormal individuals, those have less hemoglobin than the normal level according to WHO criteria of hemoglobin level classification. While, the results indicated that the proportion 2 males, those who have less than normal hemoglobin level compared to that proportion 8 females those who have less hemoglobin than the normal level. The results revealed that there is a significant (P<0.05) correlation between Hb level and gender groups. This data is finding that the proportion of cases of anemia related to iron deficiency differs among age groups. Correspondence to our study, data from Yemen and India found a higher prevalence of IDA in girls than boys. This proportion reached more than 60% among women of reproductive age versus only 15% in women aged 50 to 74. Iron deficiency anemia is the main type of anemia in younger women[8].

(Table 2) Classification of population study among hemoglobin level.

Hemoglobin level	All Sample	Male	Female
	Frequency	Frequency	Frequency
Normal	51	30 (60%)	21(40%)
Abnormal	10	2 (20%)	8 (80%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and gender			
Pearson Correlation	0.0678	0.480	0.072
P-value	0.000	0.922	0.041

(*P<0.05*) indicates a significant correlation between two factors.

(Table 3) shows the distribution of samples according to the general pallor. The results showed that account 12 students with pallor while 49students, they have not pallor of all samples. Whereas the account male and female were 3 and 9 respectively, those regarding pale skin cases have seen in this survey. The relationship between hemoglobin level and general pallor among students, the results have shown that a significant (P<0.05) correlation between Hb level and pallor skin of all samples. Whereas, there is no significant *(P>0.05*) correlation regarding male and female samples. These findings are agreed to [7], stated that, since the majority of iron in the body is used for the synthesis of hemoglobin, the most important finding of iron deficiency is anemia. In iron deficiency anemia, clinical findings secondary to anemia may be found as in all anemias or the diagnosis can be made during laboratory investigations in the absence of any clinical finding. This paleness in people with iron deficiency can appear all over the body, or it can be limited to one area, such as the face, gums, inside of the lips or lower eyelids and even the nails[9].

(Table 3) Distribution of Hb level and General pallor between samples.

General pallor	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	49	29 (60%)	20 (40%)
Yes	12	3 (30%)	9 (70%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and General Pallor			
Pearson Correlation	0.384	0.232	0.363
P-value	0.002	0.202	0.053

(*P<0.05*) indicates a significant correlation between two factors.

(Table 4) shows the distribution of samples with the pale-colored inside lower eyelids, so the results showed that about 10 students, those who have pale inside eyelid compare to other populations have normal color inside lower eyelids. The low proportion of female, who have pale-colored inside lower eyelids compared with a female, who have normal color inside lower eyelids. The results of this study revealed that, there is a significant (P<0.05) correlation between hemoglobin level and pale-colored inside lower eyelids among all samples of the study population. Whereas, a significant difference (P<0.05) between hemoglobin level and pale-colored inside lower eyelids among the samples of female but a no significant *(P>0.05*) between hemoglobin level and pale-colored inside lower eyelids among the samples of the male in the current study. This finding is closed to that pale skin and pale coloring of the inside of the lower eyelids are other common signs of iron deficiency anemia[10].

(Table 4) Study of pale-colored inside lower eyelids in the study samples

Pale inside lower eyelids	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	51	30 (60%)	21 (40%)
Yes	10	2 (20%)	8 (80%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and pale-colored inside lower eyelids			
Pearson Correlation	0.317	0.380	0.042
P-value	0.013	0.827	0.032

(*P<0.05*) indicates a significant correlation between two factors.

(Table 5) shows the distribution of mouth soreness among the study population. The results showed the majority of students haven't mouth soreness, only 7 students have complained from the soreness of the mouth, but the majority of male and female, those who haven't mouth soreness. Whereas, the result revealed that there is no significant *(P>0.05*) correlation between hemoglobin level and mouth soreness among all populations study. This finding agreed with that, low hemoglobin in iron deficiency case can cause the tongue to become pale, while lower levels of myoglobin can cause it to become sore, smooth and swollen. A sore, swollen or strangely smooth tongue can be a sign of iron-deficiency anemia. Cracks on the corners of the mouth can also be a sign[11].

(Table 5) distribution of Mouth Soreness among the study population.

Mouth Soreness	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	54	28 (52%)	26 (48%)
Yes	7	4 (57%)	3 (43%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and Mouth Soreness			
Pearson Correlation	0.032	0.089	0.053
P-value	0.807	0.629	0.784

(*P<0.05*) indicates a significant correlation between two factors.

(Table 6) shows the distribution of samples according to dizziness occurrence. The results showed the 22 students have been complained from dizziness occurrence when the majority of students were no feeling with dizziness during the study day. The majority of males haven't dizziness occurrence compared to the majority of females, those who have been complained from dizziness occurrence during a day. Whereas, the result revealed that there is no significant correlation between hemoglobin level and dizziness occurrence

among all students in this study. Headaches and dizziness could be a sign of iron deficiency. The lack of hemoglobin means not enough oxygen reaches the brain, causing its blood vessels to swell and create pressure[12].

(Table 6) Samples distribution of Dizziness occurrence.

Dizziness	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	39	26 (67%)	13 (33%)
Yes	22	6 (29%)	16 (71%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and Dizziness			
Pearson Correlation	0.245	0.097	0.070
P-value	0.057	0.599	0.720

(*P<0.05*) indicates a significant correlation between two factors.

(Table 7) shows the distribution of samples according to breath shortness. The results showed the 20 students have complained of breath shortness when the majority 41 of students were not complained, but the majority of a male were no complained with breath shortness compared to 11 females in the same case. Whereas, the result appeared there is no significant *(P>0.05*) correlation between hemoglobin level and breath shortness among all populations in this study. Shortness of breath is a symptom of iron deficiency anemia since low hemoglobin levels mean the body isn't able to transport oxygen to muscles and tissues effectively. This means your muscles won't get enough oxygen to do normal activities, such as walking[13].

(Table 7) Shortness of breath among study population.

Shortness	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	41	23 (55%)	18 (45%)
Yes	20	9 (45%)	11 (55%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and shortness			
Pearson Correlation	0.139	0.026	0.257
P-value	0.284	0.888	0.179

(*P<0.05*) indicates a significant correlation between two factors.

(Table 8) the results showed that the 29 of students have been complained from headache occurs when the 32 students have not complained, but the majority of a male was not complained about headache occurrence compared to majority 69% of female proportion, those who have been always complained from headache occurrence during the study. Whereas, the result indicated that there is no significant *(P>0.05*) correlation between hemoglobin level and headache occurrence among all populations in this study. The finding of the current study agreed that in iron deficiency cases, low levels of hemoglobin in red blood cells mean that not enough oxygen can reach the brain. As a result, blood vessels in the brain can swell, causing pressure and headaches[12].

(Table 8) Distribution of study population with of headache occurrence.

Headache occurrence	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	32	23 (70%)	9 (30%)
Yes	29	9 (31%)	20 (69%)
N	61	32 (53%)	29 (53%)
The relationship between Hb and Headache			
Pearson Correlation	0.220	0.087	0.084
P-value	0.089	0.634	0.666

(*P<0.05*) indicates a significant correlation between two factors.

In (table 9) the results appeared that the 26 students have complained from anorexia when the 35 students were normal appetite, the majority 72% of male was no anorexia compared to majority 59% of female proportion those who have been complained from anorexia during this survey. Whereas, the result revealed there is no significant *(P>0.05*) correlation between hemoglobin level and anorexia among all populations in this study. But anorexia may be related to body weight. Anorexia is an eating disorder characterized by significant and potentially health-affecting weight loss. People with anorexia voluntarily refuse to eat because they are continually concerned about their weight. The present study findings tended with that IDA is the most popular type of anemia and can occur in those with the eating disorder anorexia[14].

(Table 9) Distribution of Anorexia among the study population.

Anorexia	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	35	23 (66%)	12 (44%)
Yes	26	9 (35%)	17 (65%)
N	61	32 (53%)	29 (47%)
The relationship between Hb and Anorexia			
Pearson Correlation	0.219	0.098	0.164
P-value	0.089	0.595	0.395

(*P<0.05*) indicates a significant correlation between two factors.

(Table 10) shows the distribution of heartbeat disorder in the study population. The results showed the majority 74% of students were normal heart beating, when the 26% of students have complained about heat beat disorder, so among gender group the majority 94% of male and 52% of female proportion, those who have normal heart beating but considerable proportion 48% of female, who have been heartbeat disorder. Whereas, the result revealed that there is a significant *(P*<0.05) correlation between hemoglobin level and heartbeat disorder among all population in this study. The results of the present study matched to that, in cases of iron deficiency, the heart has to work extra hard to transport oxygen around the body. This can lead to irregular or fast heartbeats and even heart murmurs, an enlarged heart or heart failure[15].

(Table 10) distribution of study population according to Heart Beat Disorder.

Heartbeat Disorder	All Sample	Male	Female
	Frequency	Frequency	Frequency
No	45	30 (78%)	15 (22%)
Yes	16	2 (12%)	14 (88%)
N	61	32 (53%)	29 (53%)
The relationship between Hb and Heart Beat Disorder			
Pearson Correlation	0.390	0.001	0.196
P-value	0.002	0.995	0.309

($P<0.05$) indicates a significant correlation between two factors.

Conclusion and Recommendations

In this study, concluded that the prevalence of IDA among nursing and health sciences students have been measured using several assessment methods, also found a substantial prevalence of low hemoglobin levels was 16% as general. Greater proportion of female was suffering from low Hb level rather than that in male, which considered mild IDA should be appreciated according to the WHO criteria represents an 'anemia classification'. In addition, in this study found a substantial prevalence of clinical symptoms as breath shortness, dizziness, headache and heartbeat disorder. Physical signs such as general pallor, mouth soreness, pale-colored inside lower eyelids have appeared among IDA suffering students. There was evidence concerning the significant correlation between sex and IDA in students. Correspondence to our study, data from Yemen and India found a higher prevalence of IDA in girls than boys.

Anemia treatment plans often include improve dietary patterns and change eating behavior. The best diet plan for anemia includes intake foods rich in iron and other vitamins essential to hemoglobin and red blood cell production. It should also include foods that help your body absorb iron better such as animal products. It is preferable to perform a periodic detection every 6 months for the early detection and treatment of anemia before the condition.

Conflict of interest:

The authors have declared no conflict of interest and that they are solely responsible for the content and writing of the manuscript.

Author's Contribution:

Ayman Mustafa and Salem Elwahaishi: Designing of research, formulation of the plans and supervision. Ayman Mustafa and Fatima Ageel: Collection of data, performed the field experiments and Lab work. Abdalla Elgenaidi: statistical analysis of data and literature citations. Ahmed Elhamroush and Salem Elwahaishi: Helped in writing and reviewing the manuscript. All authors critically reviewed the data and the manuscript.

REFERENCES

1- The National Heart, Lung, and Blood Institute (NHLBI) September 2011 anemia Healthy Lifestyle Changes Prevent Treatment Control (Usda's).

2- World Health Organization. *Iron Deficiency Anaemia: Assessment, Prevention, and Control: A Guide for Programme Managers*. Geneva, Switzerland: World Health Organization; 2001.

3- McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B. Worldwide prevalence of anemia, WHO Vitamin and Mineral Nutrition Information System,1993-2005. Public Health Nutr 2009; 12: 444-54.

4- Powers JM, Buchanan GR. Diagnosis and management of iron deficiency anemia. Hematol Oncol Clin North Am 2014; 28: 729-45.

5- Céline Plante, Carole Blanchet1 and Huguette Turgeon O'Brien (2007) Iron Deficiency and Anemia among Women in Nunavik 20(9).

6- Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. Cell 2010; 142: 24-38.

7- Goodnough LT, Schrier SL. Evaluation and management of anemia in the elderly. Am J Hematol 2014; 89: 88-96.

8- Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med 2005; 352: 1011-23.

9- Macdougall IC. Iron supplementation in nephrology and oncology: what do we have in common? Oncologist 2011; 16: Suppl 3: 25-34.

10- Thomas DW, Hinchliffe RF, Briggs C, Macdougall IC, Littlewood T, Cavill I. Guideline for the laboratory diagnosis of functional iron deficiency. Br J Haematol 2013; 161: 639-48.

11- Schrier SL. Causes and diagnosis of iron deficiency anemia in the adults. 2014 (http://www. up-to-date .com/ contents/ causes-and-diagnosis-of-iron-deficiency -anemia-in-the-adult).

12- Johnson-Wimbley TD, Graham DY. Diagnosis and management of iron deficiency anemia in the 21st century. *Therapy Adv Gastroenterol*. 2011;4(3):177-184.

13- Van Vranken M. Evaluation of microcytosis. *Am Fam Physician*. 2010;82(9):1117-1122.

14- Goddard AF, James MW, McIntyre AS, Scott BB; British Society of Gastroenterology. Guidelines for the management of iron deficiency anaemia. *Gut*. 2011;60(10):1309-1316.

15- Skin BS, Punnonen K, Caldron PH, et al. Improved differential diagnosis of anemia of chronic disease and iron deficiency anemia: a prospective multicenter evaluation of soluble transferrin receptor and the sTfR/ log ferritin index. *Am J Hematol*. 2011;86 (11):923-927.

PREVALENCE OF VITAMIN D INSUFFICIENCY AND ITS ASSOCIATED RISK FACTORS AMONG PREGNANT WOMEN IN SELECTED CLINICS IN MISURATA, LIBYA

Hawa Omar Eltaweel, Ma. Daisery N. Garcia, Hajer Embaea, Fatima Albori, Asma Alghherrabi
Faculty of Nursing and Health Science, University of Misurata- Libya

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ABSTRACT

This study was performed to investigate the prevalence of Vitamin D insufficiency and its associated risk factors among pregnant women in selected clinics in Misurata, Libya for the month of March 2018. A total of 90 pregnant women were included in this study. a self-made questionnaire was designed to gather data. The data gathered were classified, tabulated and analyzed using descriptive statistics. Fifty five percent of the respondents had insufficient vitamin D (serum concentration 25 (OH) D level ≤ 30 ng/ml), Most of the respondents are aged 15-25 years old and are in college level, with fair skin and sometimes exposed to sunlight in the mid-day with less than one hour, sometimes use of sunscreen cream. In terms of dietary information, most of the respondents had regular intake of milk, omega 3 fish oil, Vitamin D and calcium supplements. Despite the increasing awareness of the importance of vitamin D in the recent period, and although pregnant women in Misurata hospitals are generally subjected to a course of supplements, but suboptimal levels of vitamin D is still common.

KEYWORDS: Vitamin D, insufficiency, risk factors, pregnant women.

INTRODUCTION

Maternal vitamin D insufficiency is thought to be common among pregnant women. Vitamin D supplementation during pregnancy has been suggested to protect against adverse pregnancy outcome such as pre-eclampsia, low birthweight and preterm births[1].

On the other hand, Maternal vitamin D insufficiency is associated with childhood rickets[2] and childhood wheezing[3]. Maternal vitamin D insufficiency is common in mothers with highly pigmented skin.[2]Women who used vitamin D-containing supplements had higher vitamin D status than in non-supplement users. However, vitamin D insufficiency was still evident even with supplemental use. Given the potential consequences of hypovitaminosis D on health outcomes, vitamin D supplementation, possibly at higher doses than currently available, is needed to improve maternal vitamin D status [2].

Indeed, vitamin D regulates >1000 human genes, and vitamin D receptors are found in most tissues and cells throughout the body[3]. Accordingly, in utero or early life vitamin D insufficiency has been linked to increased risk of respiratory infection,[4,5] type 1 diabetes, [3.6] multiple sclerosis,[7] schizophrenia,[3.8] and even placental development and function.[9] Vitamin D insufficiency in adults has also been linked to cardiovascular disease,[10] upper respiratory tract infection,[11] cancer,[12,13] and Cardiovascular Disease[14]. While relatively small amounts of vitamin D prevent nutritional rickets, larger doses and higher serum 25-hydroxyvitamin D (25[OH]D) levels appear necessary for optimal general health outcomes[15].

The most abundant circulating biomarker of vitamin D status is 25-hydroxyvitamin D (25(OH)D), which also has a longer half-life 25 days) compared to the active metabolite; 1,25-dihydroxyvitamin D (7 h).[16] Vitamin D is acquired in three ways; from sun exposure, diet and supplements, the greatest proportion is obtained from sun exposure. [17] however,

Vitamin D deficiency and insufficiency are highly prevalent during pregnancy in some sun-rich areas such as India[18,19], South Carolina[20], Saudi Arabia[21] . The most important source of vitamin D is the skin synthesis of the vitamin by UV B solar radiation[22]. Any process that reduces UV B photons from entering the epidermis will diminish cholecalciferol (vitamin D3) production. The skin pigment melanin absorbs UV B photons and can reduce vitamin D-3 synthesis by more than 90% [23].

Recently, vitamin D supplements have been used by high rate of pregnant women in Misurata, considering that the latitude of Misurata is (31.3478° N), this study was conducted to investigate the prevalence of vitamin D insufficiency among a sample of Libyan pregnant women in selected hospitals in Misurata. The results will reflect the possible success achieved to improve vitamin D status. Moreover, the study aimed to determine factors associated with vitamin D status during pregnancy.

MATERIALS AND METHODS

Research Design and Respondents:

The study employed a descriptive research design mainly because the present investigation is descriptive in nature. The main purpose of the study is to determine and identify the prevalence of Vitamin D insufficiency and its associated risk factors among pregnant women in selected clinics in Misurata, Libya.

Descriptive research design is appropriate for this study since it used procedures in quantitative research in which the researcher administers a survey or a questionnaire in collecting data from the respondents.

Correspondence and reprint request: Hawa Omar Eltaweel, Faculty of Nursing and Health Science, University of Misurata- Libya E-mail: hawa.biochem@gmail.com

The respondents of the study were pregnant women in selected clinics in Misurata, Libya. A total of 90 pregnant women were included in this study during the month of March 2018.

Data collection and statistical analysis:

The data needed in this study were gathered with the use of a self-made questionnaire. a questionnaire was formulated to reflect the prevalence of Vitamin D insufficiency and its associated risk factors among pregnant women. It included 6 questions and 2 tables for the respondents to answer and explained in local dialect.

A letter requesting for a permit to conduct the study was submitted to the head nurse who was subsequently approved. a permit was secured from the clinic which includes the reading of the patient's file in order to gain more information on patient's profile. The data gathered were classified, tabulated and analyzed using descriptive statistics. These were utilized to derive profiles and established frequency distributions in the presentation of the prevalence of Vitamin D insufficiency and its associated risk factors among pregnant women. The statistical tools used were tally, frequency, count, and percentage (table 1) and (table 2).

RESULTS

In total, 90 pregnant women were participants in the current study. Vitamin D insufficiency (serum concentration of 25(OH)D ≤30 ng/ml) was reported in about (55%) of them, while the rest which is about (45%) had Vitamin D sufficiency (serum concentration of 25(OH)D >30 ng/ml). vitamin D insufficiency was defined as a 25(OH)D concentration of 30 ng/mL or less[24]. (56%) of respondents were aged 15–25 years and (98%) of them are living in Misurata, (40%) in their Second trimester, moreover the majority of respondents were house wives (62%), (41%) were at the university level, and (47%) were fair skinned.

(Table 1) Profile of the Variables and lifestyle characteristics and the prevalence of vitamin D insufficiency among respondents.

Variables	respondents n (%) N=90	Vitamin D insufficiency (%) N=49
Serum Concentration 25 (OH) D Level ≤30 ng/ml (insufficiency)	49 (55%)	
>30 ng/ml (sufficiency)	41 (45%)	
Age		
15-25	51 (56%)	30 (61%)
26-35	34 (38%)	17 (35%)
36-45	4 (4%)	1 (2%)
46-55	1 (2%)	1 (2%)
Location		
Misurata	88 (98%)	49 (100%)
Other	2 (2%)	
Age of Gestation		
First trimester	22 (24%)	12 (26%)
Second trimester	36 (40%)	23 (47%)
Third trimester	32 (36%)	14(27%)
Educational Level		
College	37 (41%)	22 (45%)
Secondary	11 (12%)	7 (14%)
Primary	16 (18%)	5 (10%)
Less than primary	26 (29%)	15 (31%)
Occupation		
Retired	3 (3%)	2 (4%)
Employed	18 (20)	8 (16%)
Student	13 (15%)	6 (12%)
House wife	56 (62%)	33 (68%)
Skin color		
Fair	42 (47%)	24 (49%)
Brown	29 (32%)	15 (31%)
Black	19 (21%)	10 (20%)
Sun Exposure		
Frequently	20 (22%)	9 (18%)
Sometimes	52 (58%)	26 (53%)
Rarely	18 (20%)	14 (29%)
Time of Exposure to Sunlight		
Early morning	29 (32%)	16 (33%)
Mid-day	46 (51%)	22 (45%)
Late afternoon	15 (17%)	11 (22%)
Amount of Skin Exposure		
No skin exposure	14 (15%)	8 (16%)
Less than 1 hour	42 (47%)	23 (47%)
More than 1 hour	34 (38%)	18 (37%)
Use of Sunscreen		
Most of the time	27 (30%)	19 (39%)
Sometimes	43 (48%)	21 (43%)
Nothing at all	20 (22%)	9 (18%)

As shown in table 1 (58%) of women are exposed to the sun sometimes, while (22%) are frequently exposed and (20%) are exposed rarely, The usual time of sun exposure was mid-day (51%), with less than 1 hour of skin exposure for (47%) of respondents, furthermore (48%) use sunscreen sometimes, but (30%) apply sunscreen most of time, and the rest (22%) do not use sunscreen at all.

(Table 2) vitamin D and dietary supplements intake and the prevalence of vitamin D insufficiency among respondents.

	All respondents n (%) N=90		25 (OH) D Level ≤30 ng/ml N=49		25 (OH) D Level >30 ng/ml N=41	
	Yes n(%)	No n(%)	Yes n(%)	No n(%)	yes n(%)	No n(%)
Milk	68 (76%)	22 (24%)	31 (63%)	18 (37%)	37 (90%)	4 (10%)
Omega-3	54 (60%)	36 (40%)	23 (47%)	26 (53%)	31 (76%)	10 (24%)
Multi vitamins	43 (48%)	47 (52%)	19 (39%)	30 (61%)	24 (59%)	17 (41%)
Vitamin D	60 (67%)	30 (33%)	31 (63%)	18 (37%)	29 (71%)	12 (29%)
Calcium	49 (54%)	41 (46%)	27 (55%)	22 (45%)	22 (54%)	19 (46%)

(Table 2) represents the dietary information of Vitamin D insufficiency among pregnant mothers which includes: milk intake, omega-3 fish oil supplement, multivitamin supplement, vitamin D supplement and calcium supplement per day. it is most evident that most of respondents takes milk (76%) and various dietary supplements. (60%), (48%), (67%), and (54%) of respondents takes Omega-3, Multi vitamins, Vitamin D, Calcium supplement respectively. This study shows that (63%) of pregnant women with vitamin D insufficiency drink milk as well as (90%) of pregnant women with vitamin D sufficiency. Similarly, (47%) who takes omeg-3 supplements of insufficient group compared with (76%) in sufficient group.

Also, a lower intake of multi vitamins and vitamin D supplements was observed in the insufficient group on the contrary to the sufficient group, (39%) and (63%) of insufficient vitamin D pregnant women compared with (59%) and (71%) of sufficient ones respectively. For Calcium supplement intake, there was no difference observed between insufficient and sufficient vitamin D pregnant women.

DISCUSSION

High prevalence of vitamin D deficiency among Libyan women was reported by several studies. One study was conducted in Benghazi region found that (75%) of women had 25(OH) D < 50 nmol/l [25], another from Tripoli reported that (61%) of nursing mothers had 25(OH)D<30 nmol/l[26]. F. Faid and her colleagues investigated vitamin D intake and status level and associated factors among 455 participants in Misurata region, according to their finding women (25-64 y) were identified as the most vulnerable group with vitamin D inadequacy present in (82%)[27]. In another study which was conducted on 79 Libyan pregnant women, Albakoush and azab reported that (84.8%) of participants had a vitamin D deficiency (less than 20 ng/ml)[28] .

In this study, Vitamin D insufficiency (serum concentration of 25(OH)D ≤30 ng/ml) reported in about (55%) of participants. Although this percentage shows improvement in vitamin D status compared to other studies, Vitamin D insufficiency is still considered high in an area rich in sunlight throughout the year.

Vitamin D status is usually affected by several determinants, especially those that have an impact on the dermal synthesis rate of vitamin D, such as skin color, sun exposure, time of exposure to sunlight, amount of skin exposure and use of Sunscreen creams.

According to Mithal et al.[29] ,the prevalence of a lower level of serum vitamin D (<25nmol/L) is most common in the Middle East and is associated with women, darker skin pigmentation, limited sun exposure, higher latitude and lack of foods fortified with vitamin D. A study done in Arab populations and some other countries such as South Asia which might have similar practices suggests that skin pigmentation is probably the biggest risk factor in vitamin D deficiency regardless of the ultraviolet (UV) light exposure[30,31.32].

Although the greatest proportion of vitamin D is obtained from sun exposure [17] between approximately 09.00 and 15.00[33], Vitamin D deficiency and insufficiency are highly prevalent during pregnancy in some sun-rich areas[18-21]. The Middle East, have also shown a high prevalence of vitamin D deficiency, ranging from 50 to 97%. These findings have been explained as being mostly due to the customary clothing that covers almost the entire body[34], however hypovitaminosis D was surprisingly common in people living in sunny countries and where the body is not covered entirely, such as the European countries bordering the Mediterranean.[35,36] Regarding the application of sunscreen, Some studies have shown decrease in vitamin D with sunscreen use[37], but most have not[38, 39] This may be because the sunscreen has been applied incorrectly or irregularly or with low SPF[39].

Vitamin D is found naturally in a limited amount in just a few foods, for example, fatty fish, eggs, organ meats and UV-irradiated mushrooms. Fortified food and vitamin supplements are needed[40], There are few studies conducted in Libya that report on low vitamin D status in women and very low consumption of vitamin D supplements and vitamin-D-rich food, while there are not many fortified foods in Libya[25,26,27], This study reveals relatively high consumption of supplements rich in vitamin D, which reflect a less vitamin D insufficiency compared to other studies. Many studies have shown that the consumption of vitamin D supplements has a clear role in reducing the prevalence of deficiency, in a study conducted on 65 Saudi women for one year into three groups. The first group received only advice for healthy food, while the second group received the same advice in addition to vitamin D supplements. The third group received exercise in a sport center in combination with advice for healthy food and vitamin D supplements. Results revealed that the first group had no significant change in the level of serum vitamin D. Vitamin D level in the second group increased up to 70% of the base readings. Interestingly, vitamin D level of third group increased up to 300% of the initial readings[41]. In another study Ganji and his colleges found that Administrating vitamin D supplement for 3 months led to a significant increase in serum level of 25-hydroxyvitamin D from 10.4 ± 4.2 ng/mL to 44.0 ± 10.7 ng/mL [42]. a study was conducted on elderly people also found that users of vitamin D supplements and/or sunlamps had higher 25 (OH) D (median 54 nmol/L) than none users (median 31 nmol/L) [43].

With severe vitamin D deficiency; vitamin D supplementation may help to improve the vitamin D serum level as shown among study participants where their serum vitamin D level was elevated to sufficient range [44].

However, consumption of vitamin D supplements in pregnancy have to be in proper dose, Bodnar and others suggest that black and white pregnant women and neonates residing in the northern US are at high risk of

vitamin D insufficiency, even when mothers are compliant with prenatal vitamins and Higher-dose supplementation is needed to improve maternal and neonatal vitamin D nutriture [45].

Our study was limited by a small sample number, and lack data on the prenatal vitamin brand and dose used by our subjects. Such information would have allowed us to determine whether women were supplemented with vitamin D-2 or vitamin D-3 and the amount they received daily.

CONCLUSION

This study was conducted to investigate the prevalence on vitamin D insufficiency and its risk factors among pregnant women in selected clinics in the city of Misurata. In conclusion, over half of participants had vitamin D insufficiency which considered high in area at latitude (31.3478° N).

Despite the increasing awareness of the importance of vitamin D in the recent period, and although pregnant women in Misurata hospitals are generally subject to a course of supplements, suboptimal levels of vitamin D is still common. More studies are needed to improve the vitamin D status among pregnant women in the city of Misurata.

REFERENCES

1- De-Regil, L. M., Palacios, C., Lombardo, L. K., & Peña-Rosas, J. P. (2016). Vitamin D supplementation for women during pregnancy. *Cochrane Database of Systematic Reviews*, (1).

2- Holmes, V. A., Barnes, M. S., Alexander, H. D., McFaul, P., & Wallace, J. M. (2009). Vitamin D deficiency and insufficiency in pregnant women: a longitudinal study. *British Journal of Nutrition*, *102*(6), 876-881.

3- Tavera-Mendoza, L. E., & White, J. H. (2007). Cell defenses and the sunshine vitamin. *Scientific American*, *297*(5), 62-72.

4- Camargo Jr, C. A., Rifas-Shiman, S. L., Litonjua, A. A., Rich-Edwards, J. W., Weiss, S. T., Gold, D. R., ... & Gillman, M. W. (2007). Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *The American journal of clinical nutrition*, *85*(3), 788-795.

5- Camargo, C. A., Ingham, T., Wickens, K., Thadhani, R., Silvers, K. M., Epton, M. J., ... & NewZealand Asthma and Allergy Cohort Study Group. (2011). Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma. *Pediatrics*, *127*(1), 180-187.

6- Zipitis, C. S., & Akobeng, A. K. (2008). Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis. *Archives of disease in childhood*, *93*(6), 512-517.

7- Munger, K. L., Zhang, S. M., O'reilly, E., Hernan, M. A., Olek, M. J., Willett, W. C., & Ascherio, A. (2004). Vitamin D intake and incidence of multiple sclerosis. *Neurology*, *62*(1), 60-65.

8- McGrath, J., Saari, K., Hakko, H., Jokelainen, J., Jones, P., Järvelin, M. R., ... & Isohanni, M. (2004). Vitamin D supplementation during the first year of life and risk of schizophrenia: a Finnish birth cohort study. *Schizophrenia research*, *67*(2-3), 237-245.

9- Evans, K. N., Bulmer, J. N., Kilby, M. D., & Hewison, M. (2004). Vitamin D and placental-decidual function. *Journal of the Society for Gynecologic Investigation*, *11*(5), 263-271.

10- D'Agostino, R. B. (2008). Vitamin D deficiency and risk of cardiovascular disease. *Circulation*, *117*(4), 503-511.

11- Ginde, A. A., Mansbach, J. M., & Camargo, C. A. (2009). Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. *Archives of internal medicine*, *169*(4), 384-390.

12- Garland, C. F., Garland, F. C., Gorham, E. D., Lipkin, M., Newmark, H., Mohr, S. B., & Holick, M. F. (2006). The role of vitamin D in cancer prevention. *American journal of public health*, *96*(2), 252-261.

13- Khammissa, R. A. G., Fourie, J., Motswaledi, M. H., Ballyram, R., Lemmer, J., & Feller, L. (2018). The biological activities of vitamin D and its receptor in relation to calcium and bone homeostasis, cancer, immune and cardiovascular systems, skin biology, and oral health. *BioMed research international*, *2018*.

14- Shapses, S. A., & Manson, J. E. (2011). Vitamin D and prevention of cardiovascular disease and diabetes: why the evidence falls short. *Jama*, *305*(24), 2565-2566.

15- Vieth, R., Bischoff-Ferrari, H., Boucher, B. J., Dawson-Hughes, B., Garland, C. F., Heaney, R. P., ... & Norman, A. W. (2007). The urgent need to recommend an intake of vitamin D that is effective. *The American Journal of Clinical Nutrition*, *85*(3), 649–650.

16- Lips, P. (2007). Relative value of 25 (OH) D and 1, 25 (OH) 2D measurements. *Journal of Bone and mineral Research*, *22*(11), 1668-1671.

17- Hossein-nezhad, A., & Holick, M. F. (2013). Vitamin D for health: a global perspective. In *Mayo clinic proceedings*, *88*(7), 720-755. Elsevier.

18- Sahu, M., Bhatia, V., Aggarwal, A., Rawat, V., Saxena, P., Pandey, A., & Das, V. (2009). Vitamin D deficiency in rural girls and pregnant women despite abundant sunshine in northern India. *Clinical endocrinology*, *70*(5), 680-684.

19- Sachan, A., Gupta, R., Das, V., Agarwal, A., Awasthi, P. K., & Bhatia, V. (2005). High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India. *The American journal of clinical nutrition*, *81*(5), 1060-1064.

20- Hamilton, S. A., McNeil, R., Hollis, B. W., Davis, D. J., Winkler, J., Cook, C., ... & Wagner, C. L. (2010). Profound vitamin D deficiency in a diverse group of women during pregnancy living in a sun-rich environment at latitude 32 N. *International journal of endocrinology*, *2010*.

21- Al-Faris, N. (2016). High prevalence of vitamin D deficiency among pregnant Saudi women. *Nutrients*, *8*(2), 77.
22- Holick, M. F. (2004). Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *The American journal of clinical nutrition*, *79*(3), 362-371.
23- Clemens, T. L., Henderson, S. L., Adams, J. S., & Holick, M. F. (1982). Increased skin pigment reduces the capacity of skin to synthesise vitamin D3. *The Lancet*, *319*(8263), 74-76.
24- Evatt, M. L., DeLong, M. R., Khazai, N., Rosen, A., Triche, S., & Tangpricha, V. (2008). Prevalence of vitamin D insufficiency in patients with Parkinson disease and Alzheimer disease. Archives of neurology, 65(10), 1348-1352.
25- Omar, M., Nouh, F., Younis, M., Younis, M., Nabil, N., Saad, M., & Ali, M. (2017). Vitamin D status and contributing factors in patients attending three polyclinics in Benghazi Libya. *J Adv Med Med Res*, *24*, 1-13.
26- Benhamed, M. M., Marwan, A. G., Dekna, M. A., & Ahmad, A. A. (2017). Vitamin D levels and rickets indices among infants and their nursing mothers in Tripoli–Libya. *The Libyan Journal of Agriculture*, *22*(1).
27- Faid F., Nikolic, M., Milesevic, J., Zekovic, M., Kadvan, A., Gurinovic, M., & Glibetic, M. (2018). Assessment of vitamin D intake among Libyan women–adaptation and validation of specific food frequency questionnaire. *Libyan Journal of Medicine*, *13*(1).
28- Albakoush, A. M., & Azab, A. E. (2018). Vitamin D and Calcium Status in Pregnant Women in Western-Libya. *Advances in Biomedical Sciences*. 3(6) 122-128.
29- Mithal, A., Wahl, D. A., Bonjour, J. P., Burckhardt, P., Dawson-Hughes, B., Eisman, J. A., ... & IOF Committee of Scientific Advisors (CSA) Nutrition Working Group. (2009). Global vitamin D status and determinants of hypovitaminosis D. *Osteoporosis international*, *20*(11), 1807-1820.
30- Knoss, R., Halsey, L. G., & Reeves, S. (2012). Ethnic dress, vitamin D intake, and calcaneal bone health in young women in the United Kingdom. *Journal of Clinical Densitometry*, *15*(2), 250-254.
31- Badsha, H., Daher, M., & Kong, K. O. (2009). Myalgias or non-specific muscle pain in Arab or Indo-Pakistani patients may indicate vitamin D deficiency. *Clinical rheumatology*, *28*(8), 971-973.
32- Arabi, A., El Rassi, R., & Fuleihan, G. E. H. (2010). Hypovitaminosis D in developing countries—prevalence, risk factors and outcomes. *Nature Reviews Endocrinology*, *6*(10), 550.
33- Webb, A. R., & Engelsen, O. (2006). Calculated ultraviolet exposure levels for a healthy vitamin D status. *Photochemistry and Photobiology*, *82*(6), 1697-1703.
34- Matsuoka, L. Y., Wortsman, J. A. C. O. B., Dannenberg, M. J., Hollis, B. W., Lu, Z. H. I. R. E. N., & Holick, M. F. (1992). Clothing prevents ultraviolet-B radiation-dependent photosynthesis of vitamin D3. *The Journal of Clinical Endocrinology & Metabolism*, *75*(4), 1099-1103.
35- Mishal, A. A. (2001). Effects of different dress styles on vitamin D levels in healthy young Jordanian women. *Osteoporosis international*, *12*(11), 931-935.
36- Van der Wielen, R. P., De Groot, L. C. P. G. M., Van Staveren, W. A., Löwik, M. R. H., Van den Berg, H., Haller, J., & Moreiras, O. (1995). Serum vitamin D concentrations among elderly people in Europe. *The Lancet*, *346*(8969), 207-210.
37- Matsuoka, l. Y., Ide, l., Wortsman, j., Maclaughlin, j. A., & Holick, m. F. (1987). Sunscreens suppress cutaneous vitamin D3 synthesis. *The journal of clinical endocrinology & metabolism*, *64*(6), 1165-1168.
38- Singh, S., Jha, B., Tiwary, N. K., & Agrawal, N. K. (2019). Does using a high sun protection factor sunscreen on face, along with physical photoprotection advice, in patients with melasma, change serum vitamin D concentration in Indian conditions? A pragmatic pretest-posttest study. *Indian Journal of Dermatology, Venereology, and Leprology*, *85*(3), 282.
39- Hansen, L., Tjønneland, A., Køster, B., Brot, C., Andersen, R., Lundqvist, M., ... & Olsen, A. (2016). Sun exposure guidelines and serum vitamin D status in Denmark: The status study. *Nutrients*, *8*(5), 266.
40- Holick, Michael, F. (2007). "Vitamin D deficiency." *New England Journal of Medicine*, 357(3), 266-281.
41- Al Mulhim, A.S.; Al Mulhim, A.I.; Al Mulhim, A.A.; Al Doughan, M.N. (2015). The Effect of Exercise in Vitamin Level in Saudi Female. *Int. J. Curr. Res. Acad. Rev*., 3, 251–257.
42- Ganji, M. R., Shafii, Z., & Hakemi, M. S. (2019). Vitamin D Supplementation and Risk of Hypercalciuria in Stone Formers. *Iranian journal of kidney diseases*, *13*(1), 27-31.
43- Van der Wielen, R. P., De Groot, L. C. P. G. M., Van Staveren, W. A., Löwik, M. R. H., Van den Berg, H., Haller, J., & Moreiras, O. (1995). Serum vitamin D concentrations elderly people in Europe. *The Lancet*, *346*(8969), 207-210.
44- Kalra, P., Das, V., Agarwal, A., Kumar, M., Ramesh, V., Bhatia, E., ... & Bhatia, V. (2012). Effect of vitamin D supplementation during pregnancy on neonatal mineral homeostasis and anthropometry of the newborn and infant. *British Journal of Nutrition*, *108*(6), 1052- 1058.
45- Bodnar, L. M., Simhan, H. N., Powers, R. W., Frank, M. P., Cooperstein, E., & Roberts, J. M. (2007). High prevalence of vitamin D insufficiency in black and white pregnant women residing in the northern United States and their neonates. *The Journal of nutrition*, *137*(2), 447-452.

MUCOSAL BRUCELLA INFECTION IN HUMAN: A CASE REPORT DURING COVID-19 PANDEMIC

Eltaher Elshagmani[1,5], Abdulfattah Elturki[3,7], Yuossef Swaisi[3,7], Ebrahim El Mahjoubi[2], Mohamed Baayou[7], Mohamed Elfagieh[4], Khaled S. Ben Salah[2,6,8]

1- Department of Microbiology, National Cancer Institute (NCI), Misurata, Libya.
2- Department of Pathology, National Cancer Institute (NCI), Misurata, Libya.
3- Department of Medicine, National Cancer Institute (NCI), Misurata, Libya.
4- Department of Surgery, National Cancer Institute (NCI), Misurata, Libya.
5- Department of Microbiology, Faculty of Medical Technology, Misurata University, Libya.
6- Department of Oral Pathology and Oral Medicine, Faculty of Dentistry, Misurata University, Libya.
7- Department of Medicine, Faculty of Medicine, Misurata University, Libya.
8- Department of Oral and Maxillofacial Surgery, National Cancer Institute (NCI), Misrata, Libya.

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ABSTRACT

Brucellosis in human is an uncommon disease in Libya and usually diagnosed by serological assays. The clinical symptoms of this disease in many times are none specific and vague. This report describes a case of clinical human brucellosis in Libya. The patient was a sixteen years old girl, lives in a rural area. She was suffering from intermittent fever for several days associated with chills, mild cough, and headache. The patient had history of occasional drink of goat's raw milk during the previous two months. She had no history of travel abroad and direct animal contacts. Comprehensive physical examination revealed the patient was generally unwell, pallor and, had tenderness and mild hepatosplenomegaly. Laboratory investigations revealed that the patient had pancytopenia (low number of blood cells count) and a marked increase in C-reactive protein (CRP), Procalcitonin (PCT), Lactate dehydrogenase (LDH), Aspartate transaminase (AST). Viral screen tests were non-reactive for HCV, HBs Ag, HIV and covid-19. Provisional diagnosis on admission time was acute leukemia or aplastic anemia with common microbial infection. Empirical antibiotic (Meropenem 1 gm) was prescribed intravenously every eight hours for seven days. The patient was also given antipyretic, IV fluid hydration, and one unit of packed red blood cells. Following that, bacterial growth in aerobic blood culture was noticed and it was Gram-negative coccobacilli, non-motile. The bacterium was positive for catalase, oxidase and urease tests. It could not clearly be identified by phoenix bacterial identification system. It was provisionally diagnosed *Haemophilus* spp., but as it was able to growth on blood agar plate, it was finally diagnosed as *Brucella* spp. and that was confirmed by collection of patient serum and performing Rose-Bengal pate test (RBPT) as was significantly increased (1:640). The patient general condition was improved and blood count recovered over one week. The patient was then discharged and asked for routine follow ups during the next six months.

KEYWORDS: Brucellosis, Brucella, Human, Oral, Mucosal, Infection, Rose-Bengal test.

INTRODUCTION

Infection with Brucella is known as Brucellosis (also known as undulant fever, Malta fever and Mediterranean fever); is a disease that affects various systems in the body with a broad spectrum of symptoms and clinical signs[(1)]. Patients fail to specify these symptoms that are usually appear within two weeks of inoculation, but sometimes up to six months and the symptoms may last over a number of years if no effective treatment was provided[(2)].

Clinically, it can evolve in different degree as a subclinical, acute, subacute or chronic infection. Occurrence of this disease in human is attributed to close contact with domestic livestock and/or oral intake of contaminated dairy products, such as raw milk of infected goat without pasteurization, or soft cheese contaminated with bacteria called *Brucella melitensis* (*B. melitensis*). Also, the infection with this type of bacterium can be acquired in humans by inhalation of contaminated aerosols through the respiratory mucosa[(2)]. Accordingly, mucosa of the oral cavity is the first site of contact between Brucella and the host, and it is supplied with mucosa-associated lymphoid tissue (MALT), an immune system mechanism, belongs to the organized lymphoid structures[(3)].

These bacteria are Gram-negative aerobic, slow in growth, non-motile, non-spore forming coccobacilli and terricolous, that typically cause infection manly in sheep and goats[(4)]. They localize inside infected host's cells (intracellular), in particular within the reticuloendothelial system including spleen and liver and other organs of such type. As the causative bacterium is intracellular, recurrence of the infection is frequently observed in cases of brucellosis[(1)]. Four out of six species of Brucella are known to infect humans. They are *B. melitensis*, in goats and sheep, *Brucella abortus* (*B. abortus*) is found principally in cattle, *Brucella suis* (*B. suis*) in swine and *Brucella canis* (*B. canis*) in kennelraised dogs[(5,6)].

The human disease has a prevalence exceeding 10/100 000 population in some regions in the world with endemic nature. Annually around 500 000 new cases of brucellosis are reported worldwide[(1)]. In Asia, several countries such as China, India, Sri Lanka, and Pakistan are typical examples where the human and animal brucellosis are still widespread. Another different example is in Malaysia where animal brucellosis was reported for the first time in 1950 whereas human Brucellosis was first isolated in 2010 affecting a seven-year boy as a result of drinking an infected raw goat's

milk. However, research showed that a large proportion of seropositive patients in Malaysia were veterinarians and farmers who had history of close contact with animals as their occupation request that. Most of the cases (90%) were males with age ranging between 20 and 45 years[6]. In Africa, Brucellosis is an endemic and one of the main zoonotic diseases and its prevalence in animal can be considered as an indicator of the potential existence of infection in human.

Brucellosis in Libya has been reported in individuals, sheep and goats, cattle and camel[7]. Although the availability of significant amount of data concerning clinical manifestations of brucellosis, yet there is shortage in its geographical representation. Absence of data with good quality in the literature from Libya may theoretically represent either a lower burden of disease or a poor surveillance system for brucellosis. Here we report a case of human brucellosis infecting a sixteen-year Libyan girl. The importance of this case report lies on the following points: firstly, the relatively young age of the patient. Secondly, the challenging management of such case during the Corona virus (Covid-19) pandemic where access to hospital medical services and facilities in Libya is critical at this time.

CASE REPORT

A sixteen years old Libyan girl lives in a rural area, presented with seven days history of intermittent fever associated with chills, mild cough, headache, and without history of travelling abroad or animal contact. In the past two months, she was occasionally drinking goat's raw milk. The first complete blood count (CBC) ordered by her general practitioner showed pancytopenia (Table 1) and therefore she was referred to hematology department at National Cancer Institute-Misurata (NCI-M), Libya. On the 15th of June, 2020, she was admitted to the department of hematology with fever 39 ^{o}C, no other localizing features. Physical examination revealed unwell general condition, pallor, and tenderness over left hypochondrial area with normotensive; but no palpable lymph nodes, neck stiffness, jaundice or skin rash.

(Table 1) The haemogram results of the patient during the brucellosis.

Date	RBC x10^6/µl	HB g/dl	MCV fl	MCHC g/dl	WBC x10^3/µl	NEUT x10^3/µl	LYMPH x10^3/µl	PLATELETS x10^3/µl	ESR 1 hour
13/06/2020	3.1	9.7	92	33.9	1.6	0.6	0.9	32	35
14/06/2020	3.08	9.3	95.8	31.5	2.3	0.8	1.3	59	6
15/06/2020	2.5	7.5	96.8	30.7	2.2	0.9	1.0	37	-
16/06/2020	3.2	9.6	96.6	30.6	3.2	1.2	1.6	44	-
17/06/2020	2.9	8.8	96.6	30.9	5.0	1.9	2.7	94	-
20/06/2020	2.8	8.3	98.9	29.9	4.0	1.6	2.11	256	-
23/06/2020	2.8	8.6	104.3	29.4	3.8	1.3	2.1	409	-
11/08/2020	4.30	13.3	98.6	31.8	5.9	3.5	1.9	343	8

RBC: red blood cells, HB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, WBC: white blood cell, NEUT.: neutrophils, Lymph.: lymphocytes, ESR: Erythrocyte sedimentation rate,

Provisional diagnosis on admission time was acute leukemia or aplastic anemia depending on the acute presentation and first picture of CBC (Table 1). Septic screening was carried out and empirical antibiotic was started with meropenem (1 gm) intravenously (IV) every eight hours for seven days, antipyretic, IV fluid hydration, and one unit of packed red blood cells (PRBC) was transfused. Chest-x ray was normal, abdominal ultrasound scan showed mild hepatosplenomegaly (liver 17 cm and spleen 15.5 cm in size); while echocardiography was normal. Serological viral screen tests by enzyme-linked immunosorbent assay (ELISA) for HCV, HBs Ag, HIV and covid-19 were non-reactive (Table 2). Widal test and Coombs test were negative. C-reactive protein (CRP), Procalcitonin (PCT), Lactate dehydrogenase (LDH) and Aspartate transaminase (AST) were markedly elevated (Table 3).

(Table 2) The results serological tests of the patient during the brucellosis.

Date	HIV	HBV	HCV	Widal test	Covid19 IgG & IgM	RBPT
13/06/2020	-	-	-	-ve	-	-
14/06/2020	-ve	-ve	-ve	-	-	-
11/08/2020	-	-	-	-	-ve	+ve 1:640

HIV: human immunodeficiency viruses, HBsAg: hepatitis B virus surface antigen, HCV: hepatitis C virus, Covid19: Coronavirus disease 2019, RBPT: Rose-Bengal plate test, -: not performed, -ve: negative, +ve: positive, IgG: immunoglobulin G, IgM: immunoglobulin M.

Alanine aminotransferase (ALT) was slightly raised while vitamin B12 (VIT. B12), blood sugar (B.S) and blood urea were normal as shown in (Table 3). Hematolgoically, blood film on the 15th of June (2020) revealed leukopenia, immature myeloid cells and no blasts cells. Bone marrow biopsy showed normal hematopoietic cells with megakaryocytes hyperplasia.

(Table 3) The blood biochemistry results of the patient during the brucellosis.

Date	LDH IU/l	CRP mg/l	PCT	Urea mg/dl	Creatinine mg/dl	ALT IU/l	AST IU/l	VIT. B12	B.S mg/dl
14/06/2020	1572	-	-	37.1	0.4	49.1	187	550	96
17/06/2020	720	93.3	-	-	-	-	-	-	-

18/06/2020	-	-	12.3	-	-	-	-	-	-
11/08/2020	119	1.8	<0.1	-	-	12.3	15.6	-	-

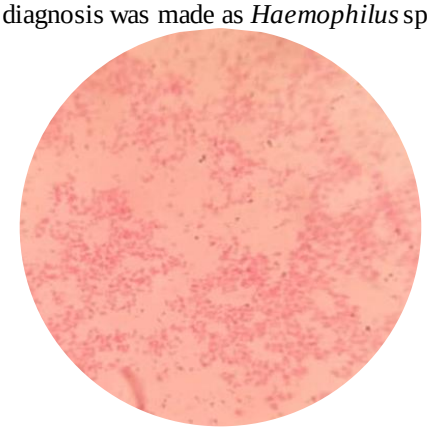
LDH: Lactate dehydrogenase, CRP: C-reactive protein, PCT: Procalcitonin, ALT: alanine aminotransferase AST: Aspartate transaminase, VIT: vitamin, B.S: blood sugar, -: not performed.

Several blood culture samples/bottles were collected and sent to the laboratory at NCI-M as follows; one bottle was collected in the admission day, and based on the presentation of fever, other two sets of blood culture bottles were collected during three consecutive days. After a period of time (4-6 days), all aerobic blood culture samples showed positive growth; while there was no growth in the anaerobic blood culture bottles.

Positive blood culture bottles were cultivated onto three plates of agar media (MacConkey, blood and chocolate). After three days of aerobically incubation at 35 ^{o}C, it was noticed a growth of small tiny colonies on both blood and chocolate; whereas, no growth was detected on the MacConkey agar even after six days (Figure 1).

(Figure 1) Three days old blood agar plate shows the colonies of *Brucella* strain isolated from the patient's blood sample.

Gram's stain showed faint gram-negative coccobacilli cells (Figure 2). Due to biochemical availability in the laboratory, only catalase and oxidase were performed and both of them were positive. Based on that, provisional diagnosis was made as *Haemophilus* spp.



(Figure 2) Gram's stain smear of three days old bacterial growth of the isolated *Brucella* spp.

on blood agar shows small faint Gram-negative coccobacilli cells.

However, the bacterium still can grow on blood agar when it was repeated several times even on fresh prepared blood agar and it was also able to grow on Mueller-Hinton agar (the colonies appeared after 72 h, see Figure 3).

(Figure 3) Three days old Mueller-Hinton agar plate shows a growth of the isolated *Brucella* spp.

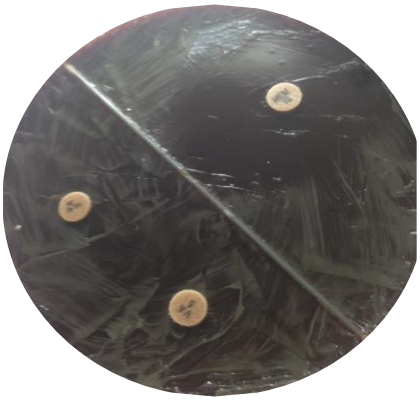
The provisional diagnosis was therefore not convincing. The isolate was sent to Zliten Central Hospital to be identified by phoenix bacterial identification system (Onco2 G-) and the bacterium was identified as *Kingella denitrificans* with confidence (96%); while antimicrobial susceptibility could not be detected. *K. denitrificans* is facultative anaerobic and β-hemolytic coccobacilli. As the isolated bacterium grew only aerobic and was non-haemloytic, *K. denitrificans* can be excluded. After a period of time that was spent to look at literatures, the final diagnosis was *Brucella* spp.

During the treatment at NCI-M, the general condition of the patient was improved with medications and blood count recovered spontaneously over one week. The patient was then discharged from NCI-M.

Recent follow-up was eight weeks later, the patient visited our hematology clinic, and she was asymptomatic with unremarkable physical examination and normal full blood count. As the bacterial isolate was finally diagnosed as *Brucella* spp.; Rose Bengal plate test (RBPT) was done and its titration in the serum was very high (1:640) which was confirming the brucellosis (Tables 2). Some other blood tests were repeated to avoid relapsing the infection and all of them were normal (Tables 1 and 3). Blood culture also was repeated and no growth was detected.

Antibiotic susceptibility test was performed using disc diffusion method according to the Clinical & Laboratory Standards Institute (CLSI). After three days of incubation, the zone of inhibition diameters were measured and they were as follows: meropenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), azithromycin (15 µg), cefotaxime (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), augmentin (30 µg) and doxycycline (30 µg) the zone of inhibition diameters were >30 mm. The zone of inhibition diameters of 15-20 mm were for cefixime (5 µg), cefuroxime (30 µg),

ceftazidime (30 µg), erythromycin (15 µg) and bactrim (trimethoprim-sulfamethoxazole, 25 µg); whereas, no zone of inhibition were detected for vancomycin (30 g) and clindamycin (2 µg) (Figure 4).



(Figure 4) Antibiotic susceptibility test for the isolated *Brucella* spp. to meropenem (MEM; zone of inhibition diameter >30 mm) and vancomycin (VA; no zone of inhibition).

DISCUSSION

Brucellosis continues to exist as one of zoonotic diseases of greatest significance and is reappearing in some areas all over the world. At present, the highest incidence of human disease is observed in different areas worldwide as in Africa, Asia, the Middle East and Latin America[2]. In Libya, many regions are endemic for brucellosis[7]. Brucellosis is caused by intracellular Gram-negative bacterium called Brucella. Human brucellosis is associated with low-rate of mortality (<5%), largely as a result of endocarditis. Yet, this disease can produce severe chronic consequences with high percentage of mortality.

The acute symptoms that appear on individuals infected with Brucella include intermittent fever, myalgia and several clinical presentations that manifest in form of splenomegaly, hepatomegaly and spondylitis[8]. Formation of abscesses in organs such as spleen, liver and lung can also be seen. Overall, the estimated proportions of 15%, 23% and 26% of cases show lymphadenopathy, hepatomegaly and splenomegaly respectively[8]. Though being uncommon, infective endocarditis is the most destructive result of brucellosis, and may necessitate to be treated surgically. Conditions such as meningitis, nephritis, leukocytoclastic vasculitis, and deep vein thrombosis are also rare results of this disease. The ocular findings associated with brucellosis usually express as optic neuritis, uveitis and papilledema[9]. The hematological manifestations commonly involve thrombocytopenia, leukopenia, and anemia[10]. Thus, brucellosis may manifest in a delicate manner which makes its diagnosis very challenging with cardiac and neurological presentations to be extremely rare. Since brucellosis is classified as a tricky infectious disease, it can imitate many diseases characterized by their ability to affect various systems in the human's body, displaying large clinical multiformity, which often results in misdiagnosis and delays in medical care, thus raising the risk of complications[1].

Mucosa of oral cavity is Brucella's first interaction point with the host and it is supplied with a mechanism of immune system connected with mucosa-associated lymphoid tissue (MALT). As a result, this bacterium should trigger an immune recognition response in this site[3]. The oral cavity is in persistent exposure to variety of pathogens such as food, microbiota, *E. coli*, *Salmonella* spp., *Brucella* spp. or air antigens and is affected mechanically by masticatory damage rendering this place of considerable hostility. Thus, the oral mucosa has mechanisms of defense and tolerance[11,12]. In the beginning, when Brucella reaches the oral cavity, it come across number of defense mechanisms such as saliva, containing elements that suppress or down regulate microbial growth, such as lysozyme, lactoferrin, nystadine, peroxidases and immunoglobulins (Ig), mainly of type A (Ig A). There is also the gingival crevicular fluid which fills the area between the teeth and the gingiva, known as the gingival sulcus. This fluid encompasses complement molecules, antibodies, neutrophils, and plasma cells.

Consequently, the combination of saliva and gingival crevicular fluid acts as a first strong barrier in the face of pathogenic microorganisms[12]. Phagocytic cells also exist in the mucosal tissue which identifies pathogens like Brucella. Phagocytic cells as dendritic cells and macrophages (antigen-presenting cells, APCs) are distributed along the specialized tissue of the oral cavity. They are capable of catching antigens and move them to the cervical lymph nodes as they are the closest regional lymph node[3]. After they have captured the oral mucosal antigens, APCs move to the lymph node (LN) to present the antigen to the lymphocytes and send the appropriate activation signal[12,13]. It has been reported that many cases with brucellosis displaying cervical lymphadenopathy (inflammation in cervical lymph nodes) that had seemingly got the infection through their ingestion of foodstuffs contaminated with the pathogen. Oral cavity, eyes, and nasal mucosa have lymphatic drainage through submandibular maxillary lymph nodes which can function as a source for Brucella and stay inert for long periods of time reach up to 50 days[3].

The patient we presented here showed some of the above-mentioned manifestations as she presented with history of intermittent fever (seven days) associated with chills, mild cough, headache pallor, and tenderness, mild hepatosplenomegaly and without history of animal contact. However, she had history of infrequent drinking of raw goat's milk. Laboratory investigations revealed that the patient had pancytopenia and a marked increase in LDH, PCT, AST, CRP levels and positive bacterial growth in blood culture. The patient was referred to NCI-M and it was supposed the patient has hematological disorders (acute leukemia or aplastic anemia). It was suspected that the patient could

have microbial infection as secondary diseases due to the fever, markedly increased of infection markers (CRP and PCT) and detection of bacterial growth in the blood culture. However, the bone marrow studies did not approve the hematological disorders. The microbial provisional diagnosis for the isolated bacterium was *Haemophilus* spp. The patient was treated with a broad-spectrum antibacterial agent (Meropenem) and the isolated bacterium was susceptible to meropenem. Interestingly, within ten days the patient completely recovered even CBC parameters was back to normal. Based on this information, the case was manifested in a delicate manner which made its final diagnosis challenging. Thus, it was thought further professional investigations were necessarily required to deeply explain the delicate manner of this case and reveal the final diagnosis. The patient was asked for routine follow up.

In the microbiology laboratory, the identical bacterial isolates were obtained from all collected blood culture bottles that were aerobically incubated. It was confirmed that it can grow on fresh blood agar and even on Mueler-Hinton agar without addition of blood or V and X factors. It was Gram-negative coccobacilli, oxidase and catalase positive. In our laboratory, it was somewhat a new experience and challenging to fully identify this isolate due to shortage in laboratory facilities. Based on that, the isolate cannot be *Haemophilus* spp; thus, the isolates were sent to another Hospital to be fully identified by phoenix bacterial identification system, but the result was inconclusive and molecular identification at that time was unavailable. It was stated that Brucella in the laboratory can be misdiagnosed and confused with other bacteria like Haemophilus, Moraxella and Ochrobactrum[14,15]. Reviewing literatures reviled that based on the above finding, Brucella cannot be ruled out and that was confirmed by positive result of urease production (Ref. ASM 2016). It was reported that identifications by biochemical testing using automated systems and manual multi-test kit, may not give the correct identification due to Brucella minimal reactivity. This conclusive result of Brucellosis then can clearly explain the first presentation of clinical symptoms in particular pancytopenia, intermittent fever, the marked increase of PCT, CRP and the obtained bacterium in blood culture.

Giving the attention to Brucella infection, the brucellosis in our area is usually detected by performing serological tests as they are fast, the most accurate and convenient, particularly in institutions other than reference laboratories[6,16]. In post Brucella infection, the high levels of Brucella antibodies can stay for several weeks or months to be normalized[6]. As by now, *Brucella* spp. cannot be ruled out, the patient should have increased level of Brucella antibodies. Therefore, in the next follow up (after 40-45 days from hospital discharge) for the patient, RBPT was performed and the detected titration of antibodies was very high (>1:640) as the normal level should be less than 1:8[16]. Moreover, the patient was not treated as it has been reported in the most of literatures due to misidentification of the isolate in the beginning; nonetheless, the patient was fully recovered. The explanation of that, meropenem could be an effective treatment for Brucellosis or the patient spontaneously recovered. Meropenem was documented to be effective treatment on experimental brucellosis[17] and can subsided the fever caused by brucellosis[18].

Due to the pandemic of Covid-19 and the associated restrictions applied by the authorities regarding the travel between the cities in order to control spread of the disease, the treating team continued to follow-up the patient from a distance over phone contact. For a period of more than three months no sign or symptoms of relapse was reported by the parents of the patient.

Diagnosis of brucellosis can be made depending on the symptoms, serological tests and blood culture accompanied by other differential diagnosis[19]. While rare, infection with Brucella must be addressed as a new cause of cardiac insufficiency, particularly in areas classified as endemic territories while it is treatable with effective antibiotic regimen[20]. Early recognition and diagnosis of Brucellosis necessitates use of several diagnostic elements including a thorough medical history, comprehensive clinical examination, and hematological assays, biochemical assays, imaging studies, microbiological tests as well as specific molecular and serological tests for Brucella detection. Several serological tests are available including serum agglutination test, Coombs test, compliment fixation test, indirect immunofluorescent antibody test and ELISA. However, RBPT has been proved as an inexpensive, rapid and successful serological test. RBPT can be done with a limited number of tools, and the findings can be interpreted macroscopically with reliable results[15]. Yet, specific tests such as IgG and IgM are required to evaluate the disease activity and determine the actual level of the antibodies.

Variation in the standard treatments has been reported. Factors such as age of the patient and pregnancy status are affecting this difference in selection of standard treatments[21]. No statistical difference has been found with respect to the form of combination therapy on the initial clinical response of human brucellosis. The preferred antibiotic regimen for treatment of infected person with brucellosis is doxycycline 100 mg *p.o.* two times daily in combination with rifampicin 450 mg once daily *p.o.* for 6 weeks[22]. However, several treatments in form of a combination of antibiotics have been also recommended as the following. Below age of 8 years, a combination therapy of trimethoprim-sulfamethoxazole and aminoglycoside[23]. Alternatively, a combination of rifampicin and trimethoprim-sulfamethoxazole for 6 weeks is required[24]. For patients with age of 8 years and older, doxycycline and rifampicin combination could be prescribed or instead a combination of rifampicin and gentamicin[25]. Rifampicin combined with ciprofloxacin has also proven successful for 4 weeks and gives the benefit of shorter treatment time[26].

CONCLUSION

This case report contributes to the comprehension of the human brucellosis, one of the more prevalent and significant zoonotic infections worldwide. Infection with Brucella has been described to have an important, disabling and sometimes persistent effect on its patients. Large delays in timely diagnosis and treatment are the product of both shortfalls in the health care system and factors related to financial and social status. Epidemiological research from regions known to be endemic with Brucella and devoid of information could enable a clearer understand of the clinical manifestations of this disorder and its acquiring hazards and present more information for developing policies. We emphasize that clinicians and microbiologists should never overlook brucellosis in the differential diagnosis of febrile diseases especially in developing countries. Traditional simple biochemical tests should not be neglected, sometime these tests still very essential in identifying extraordinary bacterium like Brucella as it could be misidentified by most of modern used biochemical identification systems. Very limited attention has been given to the oral cavity as being the first point of interaction between the body and Brucella. Since mucosa of the oral cavity is the primary location of infection, more attention should be given to the position of lymph nodes draining to the head and neck region. It is also probable that the oral route is an upcoming means of vaccination.

REFERENCES

1- Buzgan, T., et al., Clinical manifestations and complications in 1028 cases of brucellosis: a retrospective evaluation and review of the literature. Int J Infect Dis, 2010. 14(6): p. e469-78.

2- Olsen, S.C. and M.V. Palmer, Advancement of knowledge of Brucella over the past 50 years. Vet Pathol, 2014. 51(6): p. 1076-89.

3- López-Santiago, R., et al., Immune Response to Mucosal Brucella Infection. Frontiers in Immunology, 2019. 10(1759).

4- Shaqinah N, M.M., Zamri-Saad M, Hazilawati H, Jasni and S., , In vitro penetration and survival of Brucella melitensis in lymphocytic cells of goats. . . Online J Vet Res 2012. 16(3): 104-110.

5- Kang, S.I., et al., Advanced multiplex PCR assay for differentiation of Brucella species. Appl Environ Microbiol, 2011. 77(18): p. 6726-8.

6- Hartady, T., et al., Clinical human brucellosis in Malaysia: a case report. Asian Pacific Journal of Tropical Disease, 2014. 4(2): p. 150-153.

7- Gameel, S.E.A.M., et al., Prevalence of camel brucellosis in Libya. Tropical Animal Health and Production, 1993. 25(2): p. 91-93.

8- Dean, A.S., et al., Clinical manifestations of human brucellosis: a systematic review and meta-analysis. PLoS Negl Trop Dis, 2012. 6(12): p. e1929.

9- Rolando, I., et al., Ocular manifestations associated with brucellosis: a 26-year experience in Peru. Clin Infect Dis, 2008. 46(9): p. 1338-45.

10- Sari, I., et al., A multicenter retrospective study defining the clinical and hematological manifestations of brucellosis and pancytopenia in a large series: Hematological malignancies, the unusual cause of pancytopenia in patients with brucellosis. Am J Hematol, 2008. 83(4): p. 334-9.

11- Feller, L., et al., Oral mucosal immunity. Oral Surg Oral Med Oral Pathol Oral Radiol, 2013. 116(5): p. 576-83.

12- Meyle, J., et al., The innate host response in caries and periodontitis. J Clin Periodontol, 2017. 44(12): p. 1215-1225.

13- Walker, D.M., Oral mucosal immunology: an overview. Ann Acad Med Singap, 2004. 33(4 Suppl): p. 27-30.

14- Elsaghir, A.A.F. and E.A. James, Misidentification of Brucella melitensis as Ochrobactrum anthropi by API 20NE. J Med Microbiol, 2003. 52(Pt 5): p. 441-442.

15- Batchelor, B.I., et al., Biochemical mis-identification of Brucella melitensis and subsequent laboratory-acquired infections. J Hosp Infect, 1992. 22(2): p. 159-62.

16- Díaz, R., et al., The Rose Bengal Test in human brucellosis: a neglected test for the diagnosis of a neglected disease. PLoS Negl Trop Dis, 2011. 5(4): p. e950.

17- Maletskaia, O.V., [Efficacy of some new antibiotics in treating experimental brucellosis]. Antibiot Khimioter, 2002. 47(11): p. 13-7.

18- Ertem, M., et al., Brucellosis transmitted by bone marrow transplantation. Bone Marrow Transplant, 2000. 26(2): p. 225-6.

19- Villaverde, M., et al., [Febrile syndrome: myocarditis and brucellosis]. Medicina (B Aires), 1995. 55(2): p. 145-6.

20- Efe, C., et al., A rare complication of Brucella infection: myocarditis and heart failure. Intern Med, 2009. 48(19): p. 1773-4.

21- Figueroa Damian, R., L. Rojas Rodríguez, and E.S. Marcano Tochon, [Brucellosis in pregnancy: course and perinatal results]. Ginecol Obstet Mex, 1995. 63: p. 190-5.

22- Skalsky, K., et al., Treatment of human brucellosis: systematic review and meta-analysis of randomised controlled trials. Bmj, 2008. 336(7646): p. 701-4.

23- Lubani, M.M., et al., A multicenter therapeutic study of 1100 children with brucellosis. Pediatr Infect Dis J, 1989. 8(2): p. 75-8.

24- Solera, J., E. Martínez-Alfaro, and A. Espinosa, Recognition and optimum treatment of brucellosis. Drugs, 1997. 53(2): p. 245-56.

25- Joint FAO/WHO expert committee on brucellosis. World Health Organ Tech Rep Ser, 1986. 740: p. 1-132.

26- Agalar, C., S. Usubutun, and R. Turkyilmaz, Ciprofloxacin and rifampicin versus doxycycline and rifampicin in the treatment of brucellosis. Eur J Clin Microbiol Infect Dis, 1999. 18(8): p. 535-8.

ORAL SQUAMOUS CELL CARCINOMAS IN PATIENT WITH GRAFT VERSUS-HOST DISEASE FOLLOWING ALLOGENIC BONE MARROW TRANSPLANTATION. A CASE REPORT DURING MUTANT COVID-19 PANDEMIC

Khaled S. Ben Salah[1,2,3], Abdulfattah A Elturki[4,5,6], Yuossef Swaisi[4,5,6], Fatma M. Emaetig[1,7], Mohamed A. Elfagieh[8,9], Firas K. Abdulmalik[1], Yaser O. HOWAYW[10], Mohamed A. Harrama[10], Eltaher Elshagmani[11,12], Ebrahim H. ElMahjoubi[1], Matthew E. Agwae[13], AbdulHakim W. Zaggut[2]

1- Department of Pathology, National cancer institute (NCI), Misrata, Libya.
2- Department of Oral and Maxillofacial Surgery, National cancer institute (NCI), Misrata, Libya.
3- Department of Oral pathology and Oral Medicine, Faculty of Dentistry, Misrata University, Misrata, Libya.
4- Department of Medicine, Faculty of Medicine, Misurata University, Libya.
5- Department of Medicine, National cancer institute (NCI), Misurata, Libya.
6- Department of Heamatology, National cancer institute (NCI), Misrata, Libya.
7- Department of Pathology, Faculty of Medicine, Misrata University, Misrata, Libya.
8- Department of Surgical Oncology, National cancer institute (NCI), Misrata, Libya.
9- Department of Surgery, Faculty of Medicine, Misrata University, Misrata, Libya.
10- Department of Oral and Maxillofacial Surgery, Misurata Medical Centre, Misrata, Libya.
11- Department of Microbiology, National Cancer Institute (NCI), Misurata, Libya.
12- Department of Microbiology, Faculty of Medical Technology, Misurata University, Libya.
13- Cancer Research Centre, Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, 200 London Road, Liverpool L3 9TA, UK

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ABSTRACT

Aplastic anaemia is a severe haematological disorder characterized by an inadequate number of hematopoietic stem cells, resulting in pancytopenia, formed by a hypocellular bone marrow. Disorders of this nature are widely treated with haematopoietic stem cell transplantation (HSCT). A potential chronic complication following (HSCT) is the growth of secondary malignancies. Notably, patients suffering from chronic graft versus host disease (cGvHD) secondary to HSCT have been shown to be more susceptible to oral squamous cell carcinoma (OSCC). Here, we present a rare case of a 30-year-old Libyan woman treated with HSCT for aplastic anaemia, with subsequent complications of cGvHD and OSCC after few months of HSCT. These carcinomatous lesions were detected in the buccal gingiva and retromolar pad area at age of 31. The present case report emphasizes on the connection between oral cGvHD and OSCC, and the potential appearance of OSCC after HSCT at any time of patient life. Thus, closer follow-up is mandatory for all patients treated with HSCT who developed cGvHD, and efficient cGvHD prevention and therapeutic approaches are needed

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KEYWORDS: haematopoietic stem cell transplant; graft versus host disease; oral squamous cell carcinoma; OSCC.

INTRODUCTION

Aplastic anaemia is a severe haematological illness characterized by hypocellular bone marrow, leading to an inadequate amounts of hematopoietic stem cells, in turn resulting in poor erythrocyte, granulocytes and platelets production (pancytopenia)(1). In severe cases of this condition, it is typical for neutrophil and platelet counts to fall below 500/μL and 20,000/μL respectively. Aplastic anaemia appears to be associated with a geographic variation in its prevalence. For example, it is rare in countries of the west, with an average incidence of two persons per million, while appearing more prevalent across Asia and countries of the developing world(1). In Libya, only few patients have been diagnosed and registered either due to a lack of diagnostic tools or wrong diagnosis altogether. Characteristically, the cause of this disease is unknown, but a link with certain drugs, benzene exposure, insecticides, viruses, and hepatitis have been reported(2). The aetiology however generally remains unknown in half of cases of aplastic anaemia. General weakness and tiredness, contusions, nosebleed, and gingival bleeding are primary symptoms of aplastic anaemia. There is a greater risk of infections associated with complications related to persistent pancytopenia. In patients with aplastic anaemia, sepsis caused by both bacterial and fungal infections are the major reasons for expiration. Clinically, involvement of the mouth is commonly reported, but without sufficient details about frequency, risk factors and significance of these oral conditions(1). Haematopoietic stem cell transplantation (HSCT) is the treatment of choice for patients with aplastic anaemia associated with significant success rate(3,4). A frequent complication from allogeneic transplantation is Graft versus host disease (GvHD) due to an immunologic reaction from grafting immunocompetent cells to an immunodeficient, with multiple organ involvement(5). It has been reported that about 25–40% of survivors who lived for long period of time after HSCT showed GvHD(6). Skin, liver, gastrointestinal tract, lungs and eyes are the most affected body organs in systemic GvHD. However, approximately 80% of cases show oral manifestations including atrophy, erythema, lichenoid lesions and dryness and pain in the oral cavity(5). GvHD causes a significant deterioration in the quality of life of survivors and it is likely to happen when the recipient

(host) obtains a graft from an unrelated donor, or when the host or donor is older (can be paraphrased by: when there is considerable difference in the age between the donor and recipient)[7].

The survival of patients cured of their original malignancy is influenced by emergence of secondary cancers, described as a potentially significant long-term complication following HSCT. Secondary malignancies such as leukaemia in donor-derived cells, Hodgkin disease, non-Hodgkin's lymphoma and granulocytic sarcoma are frequently reported[8]. Significant danger of developing oral squamous cell carcinoma (OSCC) and squamous cell carcinoma of the skin as secondary solid tumours has been described in many recent studies although they remain less frequent[8].

We report a rare case of a Libyan female patient who was diagnosed by her haematologist in 2017 as a case of aplastic anaemia disease at the age of 28 years, complicated by GvHD after HSCT performed 28th August 2019. The importance of this case report lies on several points: firstly, the occurrence of this carcinoma as a late complication of bone marrow transplantation on a background of GvHD after a short period of time estimated totally by about one year and few months. Secondly, there has been the challenge associated with management of such a case at the time of a worldwide pandemic due to Coronavirus (Covid-19). Thirdly, it is a challenging case of a medically compromised patient with multiple systemic diseases.

CASE REPORT

A 31-year-old Libyan woman; mother of two children aged 7 year and 5 year. She presented with a history of aplastic anaemia (AA), diagnosed in 2017. She was treated with several courses of antibiotics to control infections and received blood transfusion every 3 months to correct haemoglobin level which had dropped to 5-6 g/dl with low platelet and low white blood cells counts. In July 2017, her bone marrow aspirate and biopsy showed hypoplastic bone marrow, hyperplasia of thrombocytopoiesis cells, and 20% cellularity. She was treated with steroids, erythropoietin, folic acid and danazol for 5 months. In December 18, 2017; bone marrow biopsy was performed and revealed severely hypoplastic bone marrow with 10% cellularity. Cyclosporine was therefore added to her medication for 3 months, and she had her last dose May 5, 2018. May 7, 2018, her haemoglobin level dropped to 6 g/dl, she received 1 unit Packed red blood cells (PRBC). May 21, 2018, her bone marrow, biopsy and aspirate showed unilateral hypocellular bone marrow with features compatible with Myelodysplastic syndrome (MDS) with multilineage and hypoplastic. Results of her blood investigations are shown in (table 1). Ultrasound Scan for her abdomen, Echocardiogram and Doppler did not reveal any significant findings. A diagnosis of hypoplastic MDS (Fanconi anaemia) was arrived at and she was enrolled for a reduced intensity conditioning (RIC) allogeneic bone marrow transplantation (ALLO-BMT).

(Table 1) showing results of blood investigation performed prior to HSCT.

Test	Result
Flow cytometry	No Paroxysmal nocturnal hemoglobinuria (PNH) clone
Fluorescence in situ hybridization (FISH) for MDS	Negative
Cytogenetic	Pseudodiploid clone with 1q triplication
NGS:BCOR. Chromosomal breakage test:	Positive

The patient was made to undergo ALLO-BMT with CY/FLU/ATC/TBI conditioning. 28 August 2019, an allogenic bone marrow transplant was performed, the matched donor being her younger sister aged 16 year at operation time with age difference of 14 years between the two sisters. She shared similar blood group with the donor, different RH (patient B positive, donor B negative) and same Cytomegalovirus (CMV) status, DEP test negative for the donor.

A few months post-allogenic bone marrow transplantation, the therapeutic procedure was complicated by the onset of severe cGVHD with clinical manifestations including chest infection, acute kidney injury, mucositis, epistaxis and gum bleeding, BK cystitis, mouth GVHD and skin GVHD. Post-transplant evaluation using bone marrow, biopsy and aspirate revealed unilateral normocellular bone marrow with trilineage differentiation and maturation. No morphologic evidence of significant dysplasia. Chimerism: 98% >>>97%. Bone marrow cytogenetic: normal. Repeated bone marrow; final diagnosis: bone marrow, aspirate and biopsy: slightly hypocellular bone marrow with trilineage hematopoiesis. Last chimerism: 96%. Her skin GVHD and mouth GVHD improving on tapering steroid and Cellcept 1000mg BID.

January 05. 2021, the patient was referred to us at the National Cancer Institute of Misrata where new blood investigations have been requested for her and results shown in (table 2).

(Table 2) results of blood investigations before taking the oral incisional biopsy.

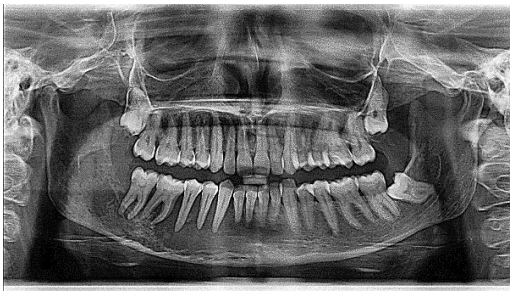
Test	Result	Test	Result
HbA1C	4.14	WBC	9.2 x 10^3/µl
CA19.9	35.1 U/ml	RBC	4.10 x 10^6/µl
CEA	3.60 ng/ml	HGB	14.3 g/dL
Blood urea	74 mg/dl	HCT	42.3%
Blood Creatinine	0.46 mg/dl	MCV	103.2 fL
Serum potassium	3.28 mmol/L	MCH	34.9 pg
Blood Sodium	138 mmol/L	MCHC	33.8 g/dL
ESR	7 mm/2hrs	PLT	139 x 10^3/µl
CRP Titer	14.0 mg/l	LYM%	19.1%
Serum Creatinine	0.7 mg/dl	MXD%	9.3%
Total bilirubin	0.6 mg/dl	NEUT%	71.6%
S.G.O.T	25 U/I	LYM#	1.8 x 10^3/µl
S.G.P.T	30 U/I	MXD#	0.9 x 10^3/µl

ALK.Phosphat	261 U/I	NEUT#	6.5 x 10^3/µl
Mg	2.1 mg/dl	RDW-SD	56.5 fL
Vitamin B12	427 bg/ml	RDW-CV	14.7%
Folic Acid	8.9	RDW	14.5 fL
HBsAg by Elisa	Negative	MPV	11.3 fL
HCV Ab by Elisa	Negative	P-LCR	35.5%
HIV by Elisa	Negative	PCT	0.16

She was first seen at the Maxillofacial Unit at Misrata medical centre in January 12. 2021 for oral evaluation. The patient complained of mouth soreness, unpleasant discharge, discomfort and pain in the posterior part of the lower right jaw. Clinical examination of the oral cavity revealed severely inflamed, erythematous and ulcerated gingiva. The buccal mucosa, bilaterally, showed red and white lichenoid-type lesions (Figure 1), and a plaque-like lesion on the right lateral side of the tongue. Examination of the teeth revealed presence of grade 3 mobility of lower molars on the right side. The possibility of epithelial dysplasia was considered. Two incisional biopsies, on the right retro-molar area and on the right buccal gingiva, were performed. Apart from the chlorhexidine mouthwash prescribed, no extra treatment was provided.

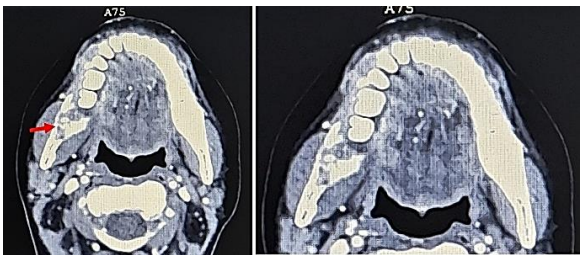
(Figure 1) intra oral photographs for the lesions at time of presentation.

The microscopic examination of the specimens showed overlying squamous epithelium with ulceration, and invasive malignant tumour composed of nests of malignant squamous epithelium. The tumour cells exhibit moderate nuclear pleomorphism with frequent mitosis, and the presence of keratin pearls. The histopathologic examination also showed presence of fibrous desmoplasia. No fungal infection was reported. The final diagnosis was moderately differentiated squamous cell carcinoma (G2). The patient presented 2 weeks later with continuing discomfort in her mouth. On examination, the oral lesions showed essentially identical features as the first examination with good healing of surgical biopsy site. Clinical examination of head and neck lymph nodes revealed no indication of their involvement. Orthopantomography (OPG) examination showed right sided invasive bone destructive lesion with osteolytic nature (Figure 2).



(Figure 2) Orthopantomograph showing presence of ill-defined osteolytic destructive lesion related to lower teeth on the right side.

Computerized tomography scan revealed presence of an ill-defined osteolytic bone destructing lesion (Figure 3), with soft tissues enhancing components on the medial and lateral mandibular aspects, measuring 5.8 x 2.7 x 1.8 cm, mainly seen at the right mandibular body, extending beyond the symphysis menti of the left side. Multiple bilateral enlarged cervical lymph nodes at levels II and III, the largest one seen at right submandibular region measuring 10 x 5 mm. The nasopharynx, oropharyngeal and laryngeal air spaces appeared normal. No glottic, supra or infra glottis mass lesions were observed. Intact cartilaginous skeleton of the larynx. Normal appearance of thyroid glands, submandibular, parotid salivary glands. Abdomen and pelvis CT scan revealed presence of right labial soft tissue mass lesion measuring 3.5 x 2.5 cm.



(Figure 3) Computerized tomography scan revealed presence of an ill-defined osteolytic bone destructive lesion with soft tissues enhancing components on the medial and lateral mandibular aspects, mainly seen at the right mandibular body.

The patient was referred to medical oncology department to provide the necessary management. Regarding medical management of this patient, immunosuppressive agents including steroid discontinued and then referred to gynaecologist to take biopsy from genital growth to exclude the presence of another potential tumour. Moreover, due to lack of oncological guidelines in the management of such a patient, and the peculiarities of the oral lesion, the patient was referred to radiotherapy department. Additionally, there was also the concern and challenge that; stoppage of immunosuppressive therapy may result in worsening of GVHD with multisystem involvement.

DISCUSSION

The risk of developing secondary cancers in HSCT patients is considerably high[6], with a greater than ten times increased frequency of oral, oesophageal and thyroid cancers[8]. It has been reported that in people who are 10 years old at the time of HSCT, the risk reaches its maximum figure and continues to be high in people of age group between 10-29 years old, while it diminishes in people who are above 30 years[3]. In patients with a primary diagnosis of aplastic anaemia and Fanconi anaemia[9], the risk of solid cancer was stated to be higher, whereas other reports have mentioned presence of higher risks for patients with a primary diagnosis of acute and chronic leukaemia[8]. The development of secondary solid tumours peaks between 8 and 9 years after HSCT[6]. In Libya, few aplastic anaemia cases have been registered and this may be due to lack of facilities for proper diagnosis. Also, stem cell transplant is not readily available locally and patients are not usually able to travel overseas for such procedures, therefore, SCC secondary to GvHD is extremely rare. In our case, the patient was aged 28 year at the time she was diagnosed with aplastic anaemia and did undergo bone marrow transplantation when she was 30 years and 4 months. Few months later she had the manifestation of GvHD and in the space of one year, starting with the appearance of the GvHD manifestation, she developed at least two OSCCs. We were not able to confirm histopathologically the clinical changes in the buccal mucosa on both sides because of her general condition at time of biopsy. The risk factors involved in the development of secondary tumours in patients treated with HSCT have been investigated by many researchers. Occurrence of new malignant lesions are broadly considered to be associated with chemotherapy agents or irradiation for pre-transplant cytotoxic therapy[6]. No relationship has been established between cytotoxic therapy and the risk of solid cancer in this case[3]. Risk factors including smoking, alcohol consumption and older age are found unrelated in patients treated with HSCT. In parallel with these data, our patient had never smoked and did not consume alcohol or other hazardous habits considered to be known risk factors. Blood investigation results of the three viral screening performed by Elisa tests were negative. Viral screening routinely done before any stem cell transplant to avoid flare up of HCV or HBV.

Chronic GvHD (cGvHD) is thought to be a possible risk factor for the development of secondary cancers after HSCT. OSCCs have been identified by many researchers in cases with widespread cGvHD with its chronic inflammation affecting the skin and mucosa[10]. Some authors[10,11] have reported two cases that developed OSCCs, one of them, having previously had cGvHD in the buccal mucosa[11]. The other OSCC appeared in the gingiva at a site of previous lichenoid mucositis. Moreover, an association between cGvHD and cancer has been demonstrated in different anatomical locations. For instance, cutaneous SCC and melanoma have developed on skin previously affected by GvHD[12]. Also, reports described pulmonary tissue previously diagnosed with GvHD-related lung disease have been affected by pulmonary mucoepidermoid carcinoma[13]. In accordance with the literature, our patient has been affected by the GvHD after receiving the HSCT therapy and developed OSCCs in at least two oral sites in addition to a labial growth as the gynaecological examination and CT scan studies revealed. It is very important to exclude SCC in genitalia because of the multifocality nature of the disease.

cGvHD of the oral cavity manifests as lesions that are clinically and histologically similar to oral lichen planus, a moderately common oral cavity condition with a limited but substantial risk for malignancy. Both conditions are of similar pathogenesis, where T-lymphocytes chronically target the epithelium of the oral cavity. Such chronic immunologic damage to the oral mucosa by T cells is thought to lead to some form of malignant transformation. For the management of cGvHD therefore, HSCT recipients must use immunosuppressive medications for prolonged periods of time, and several reports have stressed the association between the development of solid cancers and long-term use of immunosuppressive drugs[3,6]. The relationships between chronic inflammation and immunosuppression state that results from therapy is not totally clear, however, immunosuppression can hinder tissue repair in the field of chronic inflammation, such as cGvHD, thus raising the risk of malignant tumour development. In the current case, the patient has used immunosuppressive drugs and this is in agreement with the above mentioned data but the period elapsing between HSCT and the development of OSCCs as secondary solid tumours was shorter than that stated in the literature which is a relatively short period of time compared with reported peaks between 8 and 9 years after HSCT[6]. The difference in age between the donor and recipient is an important factor and as described in the literature can result in HSCT therapy failure; even if both donor and recipients are very close relatives[7]. In the present case, the patient has received the bone marrow from her younger sister because she was the only family member who was compatible. However, the fourteen years difference in age between the two sisters could be one of the reasons behind the HSCT failure and development of GvHD in our patient. Regarding medical management of this patient, immunosuppressive agents including steroid discontinued and then referred to gynaecologist to take biopsy from genital growth to exclude another potential tumour. Moreover, due to lack of oncological guidelines in the management of such patient, and the peculiarities of the oral lesion making her a poor candidate for surgery, she was referred to radiotherapy department. Additionally, the challenge is that; stoppage of immunosuppressive therapy may result in

worsening of GVHD with multisystem involvement. Furthermore, another factor that had a strong influence on our case management, beside the above-mentioned points, is the pandemic of Corona virus (Covid-19). As many surgical procedures in public and private practice has been limited to very restricted emergency cases only. Also, treatment of such case is not readily available locally and patient is not able to travel overseas because of the tough restrictions applied by the health authorities during the pandemic of Covid-19 and presence of different mutations of corona virus.

In summary, a potential risk to have oral squamous cell carcinoma is linked to HSCT patients with cGvHD at high risk at any time of their age after HSCT. The known link between GvHD of oral cavity mucosa and OSCC should be a warning to doctors to pay attention to closer follow-up and the biopsy of suspected lesions for these patients. Policies for avoiding cGvHD in patients with symptomatic GvHD should be related to the production of more successful and less carcinogenic treatments. Increased indications and patients undergoing allogenic stem cell transplant may predict an increase in the incidence of secondary cancers and needs further studies and clinical trials and development of special guidelines of management.

REFERENCES

1- Young, N.S., *Acquired aplastic anemia*. Jama, 1999. 282(3): p. 271-8.

2- Brown, K.E., et al., *Hepatitis-associated aplastic anemia*. N Engl J Med, 1997. 336(15): p. 1059-64.

3- Curtis, R.E., et al., *Impact of chronic GVHD therapy on the development of squamous-cell cancers after hematopoietic stem-cell transplantation: an international case-control study*. Blood, 2005. 105(10): p. 3802-11.

4- Georges, G.E. and R. Storb, *Hematopoietic stem cell transplantation for acquired aplastic anemia*. Curr Opin Hematol, 2016. 23(6): p. 495-500.

5- Eggleston, T.I., V.B. Ziccardi, and H. Lumerman, *Graft-versus-host disease. Case report and discussion.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1998. 86(6): p. 692-6.

6- Demarosi, F., et al., *Squamous cell carcinoma of the oral cavity associated with graft versus host disease: report of a case and review of the literature.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2005. 100(1): p. 63-9.

7- Bauters, T., et al., *Highly effective treatment with tacrolimus ointment in an adolescent with oral graft-versus-host disease*. Pharm World Sci, 2010. 32(3): p. 350-2.

8- Kolb, H.J., et al., *Malignant neoplasms in long-term survivors of bone marrow transplantation. Late Effects Working Party of the European Cooperative Group for Blood and Marrow Transplantation and the European Late Effect Project Group*. Ann Intern Med, 1999. 131(10): p. 738-44.

9- Deeg, H.J., et al., *Malignancies after marrow transplantation for aplastic anemia and fanconi anemia: a joint Seattle and Paris analysis of results in 700 patients.* Blood, 1996. 87(1): p. 386-92.

10- Otsubo, H., et al., *Gingival squamous cell carcinoma in a patient with chronic graft-versus-host disease*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1997. 84(2): p. 171-4.

11- Abdelsayed, R.A., et al., *Oral precancerous and malignant lesions associated with graft-versus-host disease: report of 2 cases*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2002. 93(1): p. 75-80.

12- Lishner, M., et al., *Cutaneous and mucosal neoplasms in bone marrow transplant recipients*. Cancer, 1990. 65(3): p. 473-6.

13- Sánchez, J., et al., *Bronchial mucoepidermoid carcinoma after allogeneic bone marrow transplantation*. J Clin Pathol, 1997. 50(11): p. 969-70.

REDUCING THE RISK OF URETHROCUTANEOUS FISTULA WITH MODIFIED SNODGRASS HYPOSPADIAS REPAIR: A RETROSPECTIVE STUDY IN MISURATA

Salem Swieb[1,2], Omar Alhaddad [1,2]

1- Faculty of Medicine, Misurata University.
2- Alnokhba Hospital - Misurata.

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ABSTRACT

The Snodgrass surgical procedure is one of the methods for hypospadias repair. Currently it is the most commonly used hypospadias repair technique, especially for distal hypospadias. It has resulted in significant improvement in the outcome of hypospadias repair. Multiple modifications to the original procedure are introduced, which may further limit and reduce the risk of complications. In this paper, we review our experience with the modified technique with respect to postoperative complications and outcome, with emphasis on fistula formation. We reviewed the results of 61 cases treated by Snodgrass urethroplasty by the same surgeon, between August 2017and May 2019 at Alnokhba hospital in Misurata-Libya. We divided the patients into three groups, we used in each group a different surgical method. The three methods differed in the type of flap used. In group A (20 boys), the neourethra was covered by a preputual flap. In group B (23 boys), the neourethra was covered by a lateral dartos flap. In group C (18 boys), the neourethra was covered by double flap formed by lateral dartos flap and preputial flap. After tubularization of the urethral plate and a circumferential incision proximal to the coronal sulcus from each edge of the urethral plate, the penile skin was degloved from 1 cm proximal to the hypospadiac meatus in all groups. A total of 61 boys underwent repair by at a mean age of 3.98 years (range 4 months to 11 years). At follow up 3 months, 8 patients had urethrocutaneous fistula as a complication (13.1%), in group A, fistula occurred in 4 cases (20%), in group B, it occurred in 3 patients (13%), while in group C, it occurred in only one patient (5.5%). Snodgrass procedure has markedly improved the outcomes for management of hypospadias. The modifications to the Snodgrass hypospadias repair described combined with proper patient selection permit a high rate of success with minimal complications. Use of a double flap (lateral dartos flap and preputial flap) to cover the neourethra in Snodgrass urethroplasty reduce the rate of fistula formation.

INTRODUCTION

Hypospadias is one of the most common congenital abnormalities of the male genital system. The reported incidence in the USA in 2001 was 1 per 200-300 live male births,[1] while the rate in the Netherlands in 2002 was 3 per 1000 live male births.[2] It is characterized by the abnormal position of the urethral meatus on the ventral penile surface and it is usually associated with ventral curvature of the penis (chordee). Hypospadias causes psychological problems for patients and their parents, in addition to the functional problems. Many techniques have been described for repairing hypospadias, but none was considered the standard method. In 1994, Snodgrass described tubularized incised plate (TIP) urethroplasty for distal penile hypospadias repair. It was subsequently also applied to proximal hypospadias, with encouraging results.[3,4] Snodgrass introduced the longitudinal split of the urethral plate which represents significant progress in urethral plate-preserving surgery, allowing tension-free tubularization of the urethral plate to form a neourethra of adequate size. The technique is now widely accepted.[5] The principal steps are a deep longitudinal incision of the urethral plate to allow tubularization, and addition of a layer between the neourethra and the overlying skin to avoid urethrocutaneous fistula.[6, 7]

The surgical goals of hypospadias repair are formation of a hairless urethra of uniform caliber and adequate size, attaining a fully straight penis, positioning of the external meatus at the tip of the glans and normal penile appearance with minimum complications. Although, complications such as fistula, meatal stenosis, urethral flap necrosis and dehiscence are still encountered.

Surgeons introduce some modifications to the original procedure in order to minimize the complications. The distal limit of the deep longitudinal incision may be either the mid-glans or the tip of the glans. The covering flap of the neourethra is usually raised from the preputial skin; however, this may result in penile torsion and devascularization of the preputial skin that is often used in reconstruction of the penile skin.[8] A ventral dartos flap has been used to cover the neourethra aiming to avoid such complications. Despite these variations, complications of hypospadias repair, such as fistulae, urethral stricture, meatal stenosis, penile torsion, persistent chordee, infections and wound dehiscence, are still reported.[9] In this paper, we describe our experience with modi-

Correspondence and reprint request:
Salem Swieb.
Department of surgery, Faculty of Medicine,
Misurata University- Libya.
E-mail: s.swieb@med.misuratau.edu.ly.

fied technique in performing Snodgrass TIP urethroplasty with respect to postoperative complications and outcomes, with emphasis on urethra-cutaneous fistula formation.

MATERIALS AND METHODS

We reviewed the results of Snodgrass TIP uretheroplasty in hypospadias repair performed by one surgeon, between August 2017 and May 2019 at Alnokhba hospital in Misurata- Libya.

Cases of hypospadias with severe chordee and patients who had undergone previous urethroplasty were excluded. Distal hypospadias in 61 boys aged 4 months to 11 years were included, with data on the demography of the patients, type of hypospadias, presence and degree of chordee and the surgical outcome and follow-up for fistula formation. All operations were performed with the patient in the supine position under general anesthesia A stay suture was placed on the dorsal side of the glans for handling, and the urethral plate was outlined at a width of 6–8 mm.

We divided the patients into 3 groups, we used in each group a different surgical method. The three methods differed in the type of flap used.

In all groups, the urethral plate defining incisions and the sagittal deep longitudinal incision were extended from the posterior edge of the hypospadiac meatus to the mid-glans

In group A (20 cases), the neourethra was covered by a dorsal preputual flap. In group B (23 cases), the neourethra was covered by a lateral dartos flap. In group C (18 cases), the neourethra was covered by combination of lateral dartos flap and preputial flap. In all groups, the urethral plate was tubularized over an 8-10 Fr fenestrated silicone catheter (depending on the child's age) with a continuous 4-0 vicryl absorbable suture to create the neourethra, and the catheter was left for 5 days postoperatively. The glandular wings were approximated by a 4-0 vicryl absorbable suture, and the distal ends were fixed to the underlying neourethra at 5 and 7 o'clock with the same type of suture. After the completion of repair, a urethral stent was fixed to the glans penis with a 3/0 silk suture. All patients were admitted to the hospital postoperatively and usually discharged at the 3rd day postoperatively. Urethral catheter was removed 5 days after operation. A slit, vertically oriented, oval meatus and a conical glanular shape with a direct urinary stream were the criteria for good results.

Data were collected and entered into the computer with the SPSS program.

RESULTS

The mean age of the 61 cases was 3.98 years (range 4 months to 11 years). The positions of the urethral meatus in the sample and in each group are shown in (table 1). An adequately functioning neourethra with a slit-like meatus at the tip of the glans was achieved in 53 (86.8%) patients.

(Table 1) types of hypospadias and number of cases studied.

Coronal	14
Subcoronal	29
Peno-scrotal	8
Mid-shaft	10

On follow-up after 3 months from operation, urethro-cutaneous fistula as a complication found in 8 patients, at a rate of 13.1% (table 2). In group A, fistula occurred in 4 cases (20%), in group B, it occurred in 3 patients (13%), while in group C, it occurred in only one patient (5.5%). A lower rate of fistula formation occurred with group C, in which we used double flap (lateral dartos flap and preputial flap).

(Table 2) percentage of fistula formation in each group of patients.

	Group A	**Group B**	**Group C**	**Total**
Number of cases	20	23	18	61
Fistula formation (%)	4 (20%)	3 (13%)	1 (5.5%)	8 (13.1%)

In correspondence with type of hypospadias, no urethrocutaneous fistula occurred with coronal hypospadias (0%), while fistula occurred in 3 cases with subcoronal type (10.34%), 3 cases with peno-scrotal type (37.5%) and 2 cases with mid-shaft type (20%). (table 3)

(Table 3) percentage of fistula formation within each type of hypospadias.

	Coronal	**Subcoronal**	**Peno-scrotal**	**Mid-shaft**	**Total**
Fistula	0 (0%)	3 (10.34%)	3 (37.5%)	2 (20%)	8
No fistula	14 (100%)	26 (89.66%)	5 (62.5%)	8 (80%)	53
Total	14	29	8	10	61

DISCUSSION

There are multiple variations in surgical techniques introduced by surgeons to modify Snodgrass procedure aiming to improve outcomes and decrease complications. Most common complications are urethra-cutaneous fistula, meatal stenosis, penile torsion and wound dehiscence. We will discuss fistula formation in our study and review literature for comparison. The interposition of a barrier layer (flap) between the neourethra and the overlying skin is an important part of the Snodgrass repair, its role is to decrease the rate of urethra-cutaneous fistula formation. The most

popular flap used is the preputial flap; however, mobilization and ventral transposition of the flap around one side of the penile shaft may lead to penile torsion, especially if the flap is of inadequate length and laid on with tension. Moreover, dissection of the flap may affect the blood supply to the dorsal skin, which is often used for re-surfacing closure, and may thus predispose to skin loss and failure of the repair.

To avoid penile torsion, a modification of the way in which the preputial flap is immobilized has been described.[10] A window is created in the flap at the midline, and the penile shaft is pulled through it in order to transfer the dartos flap ventrally over the neourethra. The size of the flap may, however, be inadequate to cover the repair when the ventral skin is deficient, and another modification in flap creation was described, which is to raise the ventral dartos flap to cover the neourethra. This technique was claimed to be associated with a low fistula rate and easier harvesting and mobilization of the flap to cover the neourethra[7,11]. Jayanthi[12] reported statistically significantly higher rates of fistula formation with preputial flap than that with lateral dartos flap. He found that the lateral dartos flap is usually easily raised and mobilized to the midline. It is also a good option in cases in which the child has been circumcised before uretheroplasty, as there is no preputial flap.

In our study, we used three different modified techniques for Snodgrass procedure, to cover the neourethra we used preputial flap, lateral dartos flap and combined double flap (preputial and lateral dartos), we found better outcomes with double flap technique and less urethrocutaneous fistula formation. Coronal hypospadias had the best outcome with no cases of fistula formation, while percentage of cases that had fistula complication increased the higher the degree of hypospadias. However, this technique (combined double flap) can be used only in selected patients, it cannot be used in already circumcised patients. Another factor that detrermines the selection of the type of flap is the position of external urethral meatus, Mid-shaft and subcoronal hypospadias are more suitable for combined double flap technique.

In (table 4) a review of other modifications of the tissue covering TIP uretheroplasty and the rates of fistula formation published in the literature.

(Table 4) types of supportive tissue covering TIP uretheroplasty and rates of fistula formation.

Tissue covering TIP repair	Reference (No.)	Date	No. of patients	No. of fistulas (%)
Lateral dartos flap	Al-Hunayan et al.[7]	2003	83	4 (5)
De-epithelialized preputial flap	Jayanthi[12])	2003	110	1 (1)
De-epithelializied preputial flap	Baccala et al.[13]	2005	101	2 (2)
Para-urethral dartos flap	Mustafa[14]	2005	15	1 (7)
Double dartos flap	Bakan and Yildiz[15]	2007	45	0
Combined Mathieu and Snodgrass	Elganainny et al.[16]	2010	101	8 (7.9)
Modified preputial flap	El-Kassaby et al.[17]	2012	764	16 (2)

Another topic of controversy in TIP urethroplasty is the use of a urethral stent. Proponents of stenting argue that it keeps the dorsal midline incision stretched open and limits premature healing, which would obviate the benefit of the dorsal incision.[12] In descriptions of cases with no stenting, however, no cases of urethrocutaneous fistula, urethral stricture or meatal stenosis have been reported[18], We stented all patients for 3-5 days, which allowed drainage of the urinary bladder. It also helps hemostasis, reduces post-operative bleeding and in the same time this short period, avoids the problem of catheter blockage, bladder irritation and long hospital stays.

The retrospective nature of this study limits its generalization, and a prospective comparative study is recommended.

CONCLUSION

Use of preputial- lateral dartos combined double flap in Snodgrass urethroplasty reduce the rate of fistula formation. The use of a stenting catheter should not exceed 5 days post-operatively in order to avoid its complications.

REFERENCES

1- L.S. Baskin, T. Colborn, K. Aimes Hypospadias and endocrine disruption: is there a connection? Environ Health Perspect, 109 (2001), pp. 1175-1183.

2- F.H. Pierik, A. Burdorf, J.M.R. Nijman, *et al.* A high hypospadias rate in the Netherlands Hum Reprod, 17 (2002), pp. 1112-1115.

3- Z. Brekalo, A. Kvesić, H. Nikolić, D. Tomić, V. Martinović Snodgrass' urethroplasty in hypospadias surgery in Clinical Hospital Mostar-preliminary report Coll Antropol, 31 (1) (2007), pp. 189-193.

4- W. Snodgrass Tubularized incised plate uretheroplasty for distal hypospadias J Urol, 151 (1994), pp. 464-465

5- W. Snodgrass Changing concepts in hypospadias repair Curr Opin Urol, 9 (1999), pp. 513-516.

6- Y. Zhou, J. Lu, G. Takarashi Snodgrass procedure for primary hypospadius repair J Urol, 9 (2002), pp. 215-218.

7- A.A. Al-Hunayan, E.O. Kehinde, M.A. Elsalam, R.S. Al-Mukhtar Tubularized incised plate urethe-

roplasty: modification and outcome Int Urol Nephrol, 35 (2003), pp. 47-52.
8- A.W. El-Kassaby, A.M. Al-Kandari, T. El-Zayat, A.A. Shokeir Modified tubularized incised plate urethroplasty for hypospadias repair: a long-term result of 764 patients J Urol, 71 (2008), pp. 611-615.
9- W. Snodgrass, M. Koyle, G. Manzoni, R. Hurwitz, A. Caldamone, R. Ehrlich Tubularized incised plate repair for proximal hypospadias J Urol, 159 (1998), pp. 2129-2131.
10- M. Samuel, S. Capps, A. Worthy Distal hypospadias: which repair? Br J Urol Int, 90 (2002), pp. 88-91.
11- T. Soygur, N. Arikan, A.E. Zumrutbas, O. Gulpinar Snodgrass hypospadias repair with ventral based dartos flap in combination with mucosal collars Eur Urol, 47 (2009), pp. 879-884.
12- V.R. Jayanthi the modified Snodgrass hypospadias repair: reducing the risk of fistula and meatal stenosis J Urol, 170 (2003), pp. 1603-1605.
13- A.A. Baccala Jr, N. Detore, J. Ross Modified tubularized incised urethroplasty (Snodgrass) for hypospadias repair Urology, 66 (2005), pp. 1305-1306.
14- M. Mustafa the concept of tubularized incised plate hypospadias repair in different types of hypospadias
Int Urol Nephrol, 37 (2005), pp. 89-91.
15- V. Baken, A. Yildiz Dorsal double layer dartos flap for preventing fistulae formation in the Snodgrass technique. Urol Int, 78 (2007), pp. 241-244.
16- E.O. El Ganainy, Y.M. Abdelsalam, M.M. Gadelmoula, M.M. Shalaby Combined Mathieu and Snodgrass urethroplasty for hypospadias repair: a prospective randomized study Int J Urol, 17 (2010), pp. 661-665.
17- A.W. El-Kassaby, A.M. Al-Kandari, T. Elzayat, A.A. ShokeirModified tubularized incised plate urethroplasty for hypospadias repair: a long-term result of 764 patients Urology, 71 (2008), pp. 611-615.
18- S. Turial, J. Enders, V. Engel Stent free tabularized incised plate repair of distal and mid-shaft hypospadias irrespective of age Eur J Surg, 21 (2011), pp. 163-170.

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