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European Journal of Therapeutics aims to contribute to the international literature by publishing original clinical and experimental research articles, short communication, review articles, technical notes, and letters to the editor in the fields of medical sciences. The journal's target audience includes researchers, physicians and healthcare professionals who are interested or working in in all medical disciplines.

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

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Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

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Original Research

Evaluation of Corneal Histopathologic Changes in Rabbits Due To Topical Mitomycin C Application in Different Doses and Periods

Kemal Yar, Altan A. Ozcan, Yurdun Kuyucu, Seyda Erdoğan, Sait Polat
Çukurova University School of Medicine, Adana, Turkey

ABSTRACT

Purpose: To evaluate the histopathologic changes in the cornea due to topical mitomycin C (MMC) application at various doses and administration periods.

Materials and Methods: The study group consisted of 35 albino rabbits with a mean age of 6 months. The animals were divided into the 7 groups, each comprising 10 eyes of five rabbits. Five rabbits were used as the control group. Other subjects were divided into the 2 groups according to the application period. The first 3 groups subsequently underwent 0.2, 0.4, and 0.8 mg/mL mitomycin C application 6 times at weekly intervals. Groups 4, 5 and 6 underwent MMC application at the same doses 12 times. The control group underwent topical physiologic serum application. After the last treatment, the rabbits were sacrificed and enucleation was performed .

Results: Light and electron microscopic examinations of mitomycin C treated animals revealed different histopathologic changes in the corneal epithelium and endothelium according to various doses and administration periods.

Discussion: Topical mitomycin C has toxic effects on the cornea related to dose and period and is known to be more toxic, especially in increased doses.. In this study, we identified the most effective dose without side effects. Long-term treatment with MMC at low doses is more advantageous than short-term treatment at high doses.

Keywords: Topical mitomycin C, Cornea, Toxic effect

INTRODUCTION

Mitomycin C (MMC) is a chemotherapeutic agent with antifibrinolytic and antineoplastic features, derived from Streptomyces caespitosus (1). It is used topically for the treatment of ocular surface tumors, glaucoma, pterygium, lacrimal canal, and ocular cicatrization. Moreover, this drug utilized as an adjuvant therapy after corneal refraction surgery and has a limited use in vernal keratoconjunctivitis, lacrimal drainage system tumors, orbital implant, proliferative vitreoretinopathy and cataract surgery.

Generally MMC is used topically for a short period in patient undergoing photorefractive keratectomy (PRK) for myopia to prevent haze formation, or it may be used for long periods in patient with ocular surface tumors, however, it may cause side effects. Because of its serious and irreversible side effects, such as scleral melting and limbal stem cell deficiency, the application form, period and dose vary according to physician (2). In the present study, we aimed to evaluate the histopathologic changes in the cornea of rabbits caused by treatment with topical MMC at various doses and application periods and to determine the most effective dose without toxic effects to prevent its side effects.

MATERIAL METHOD

The study protocol was approved by the Animal Ethics Review Committee of Medical Faculty. The study group comprised 35 New Zealand albino rabbits weighing 2961 ± 424,7 (2020-3860) g with a mean age of 6 months. The animals were divided into the seven groups, each consisting of 10 eyes of five rabbits. Five rabbits were used as a control group (Group 0), undergoing topical physiologic serum application in weekly intervals. Groups 1, 2 and 3 underwent 0.2, 0.4 and 0.8 mg/mL MMC (Kyowa® flacon), respectively, 6 times at weekly intervals (1 week with and 1 week without drop application). Groups 4, 5 and 6 underwent MMC at the same doses 12 times in weekly intervals similar to that of the topical MMC treatment model. Tumor cells with proliferative features were more sensitive to this type of therapy, whereas normal cells had time for repair. Thus, the intensity of adverse effects is thought to be less (Table 1). After the final treatment, all the rabbits were sacrificed, and anucleation was performed thereafter.

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RESULTS

Light microscopic examination of the control group's cornea revealed a regular arrangement (Figure 1); furthermore, increase in the number of epithelial cells and epithelial thickening, as well as cellular loss were noted (Figure 2).

Cornea endothelium showed a regular arrangement and insignificant cytoplasm (Figure 3); endothelial cells thinning, superficial microvillus loss, and cytoplasmic vacuolization, cellular loss were the most common changes observed in the endothelium (Figure. 4).

These changes were found to be greater according to the increase in dose and application period. Increase in dose was found to be more effective in the development of these findings.

Light microscopic evaluation was graded as follows:

- 0 (none), 1 (mild), 2 (intermediate), and 3 (intensive)
- Evaluation of the affection ratio of the groups revealed that dose was a more effective parameter than the application period (Table 2).

Electron microscopic examination of the corneal epithelium revealed nonkeratinized squamous epithelial cells and, short microvillus at the apical surface of the corneal epithelial cells (Figure 5). Furthermore, the following findings were observed:

Table 1. The Properties of the groups

	Number of eyes (n)	Duration	Number of cures
Group 0 (Control)	10	3 months	6
Group 1	10	3 months	6
Group 2	10	3 months	6
Group 3	10	3 months	6
Group 4	10	3 months	6
Group 5	10	3 months	6
Group 6	10	3 months	6

Figure 1. Regular arrangement of cornea epithelium and cytoplasm was observed in group 0 (control) (hematoxylin and eosin staining x400).

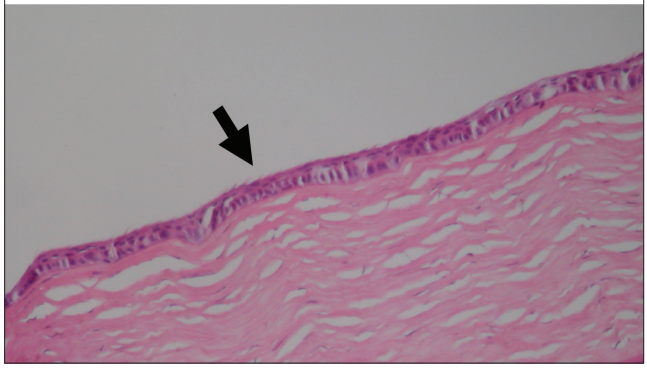


Figure 2. Increase in the linear arrangement of the cornea epithelium in group 6 (dose :8 mg/mL, treatment duration: 6 months. and number of cures:12) (hematoxylin and eosin staining x400).

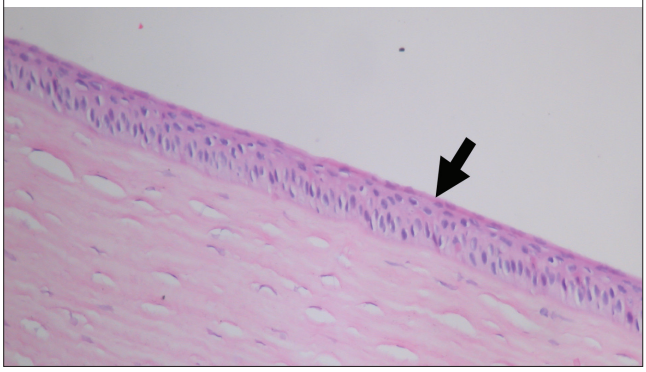


Figure 3. In group 0, cornea endothelium presented with a regular arrangement and insignificant cytoplasm (hematoxylin and eosin staining x400).

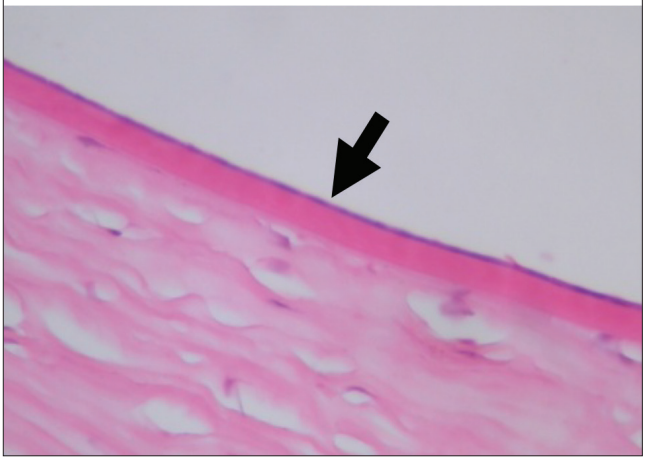


Figure 4. Vacuolar degeneration was observed in the cytoplasm of the corneal endothelium in group 6 (dose : 0.8 mg/mL, duration of treatment: 6 months, and number of cures:12) (hematoxylin and eosin staining x400).

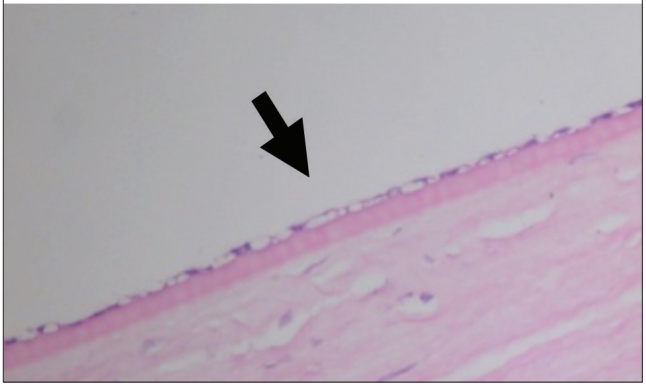


Figure 5. In group 0 (control), the corneal epithelium comprised nonkeratinized squamous epithelial cells. Short microvilli at the apical surface of the corneal epithelial cells (arrows) and intercellular binding complexes (white arrows) can be seen. Nucleus (N).

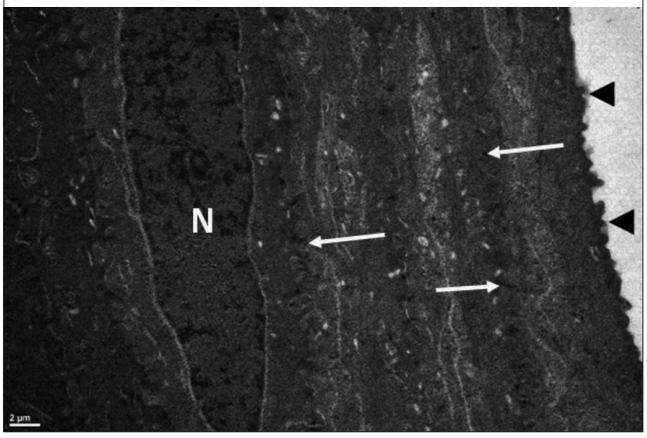


Figure 6. Cornea of group 6 (duration of treatment: 6 months and dose 0.8 mg/mL). Intercellular expansion presenting with edema inthe cornea superficial epithelium (white arrows). Nucleus (N).

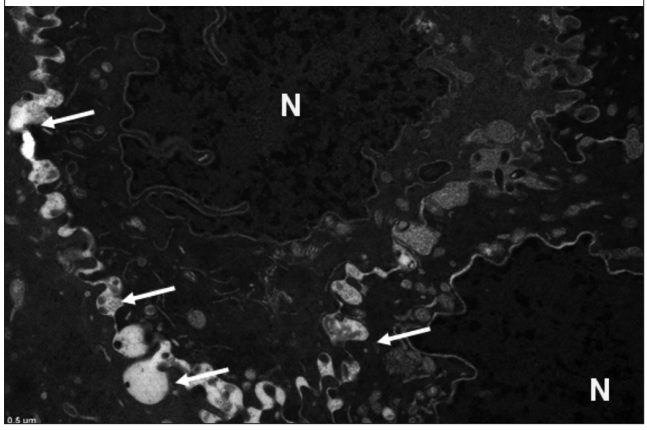


Table 2. Increase in affection due to increased dose and administration period and effect of the administration of a higher dose.

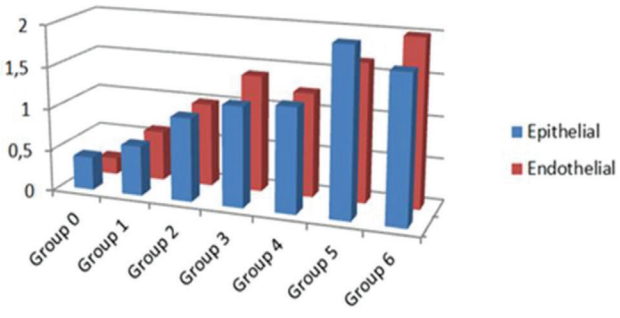


Figure 7. Endothelial cell layer (En) of group (control). Microvilli at the apical surface of the endothelial cells (black arrow). Nucleus (N), Descemet membrane (D)

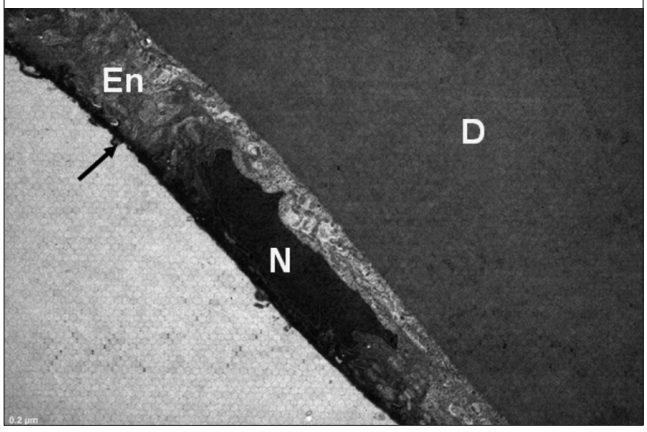
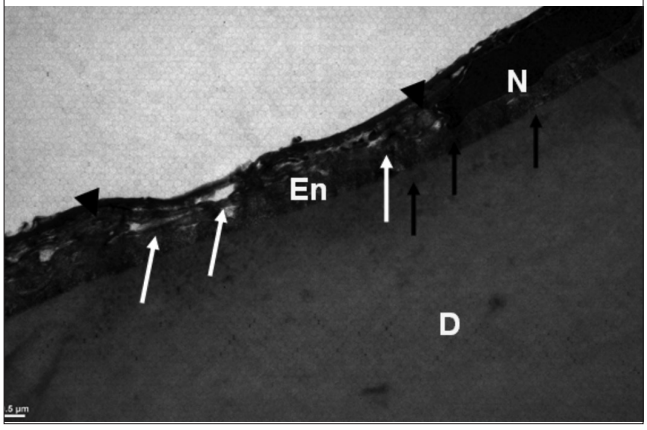


Figure 8. Cornea of group 6 (duration of treatment: 6 months and dose: 0.8 mg/mL). Extensive expansion (white arrow) between the endothelial cells (En) presenting with edema. Decrease in the numberof microvilli on the apical surface of the endothelial cells (arrows) and mild vacuolization (black arrows). Nucleus (N), Descemet membrane (D)



expansion of the intercellular area due to edema, presence of electrodense epithelial cells, decrease in intracellular binding complexes, and increase in intercellular distance. Increase in the amount of chromatin in the cell nuclei, deep indentations, expansion in the mitochondria of the cytoplasm and vacuolization were frequent findings. Except for mild expansion in fibroblasts, stroma was found to be normal (Figure 6). The endothelial cell layer and microvilli at the apical surface of the endothelial cells were also normal (Figure7). Thinning in corneal endothelial cells, superficial microvillus loss, vacuolization in cytoplasmic were common findings (Figure 8). These findings varied according to the dose and application period of MMC.

DISCUSSION

MMC is a preferred therapeutic agent in ophthalmological disorders due to its antineoplastic and antifibrotic features. It is wide-

ly used in filtering glaucoma surgery because of its antifibrotic effects, and it is used in ocular surface tumors and pterygium surgery because of its antineoplastic effects. However, there is no consensus regarding the dose and application period of MMC because of its irreversible side effects on normal cells.

Mohan and co-workers used the terminal deoksiribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) system to determine the effects of the MMC application period and concentration of MMC on corneal apoptosis. They observed a significant increase in the TUNEL- positive stained cells high doses, however, the increase due to long-term treatment was not statistically significant (2). Furthermore, Mohan and co-workers detected maximal apoptosis at the 4th hour following photoreactive keratectomy (4)

Gharaee et al. reported that the administration of 0.02 % MMC over the ablated area for 5 s for each diopter of spherical equivalent in PRK can affect the morphology of the endothelial cells, but it does not change the cell density (5).

Zare et al. reported after a 6 month follow up period that there is no significant changes in the endothelial cell density and morphology of the cornea after the administration of topical MMC during PRK (6).

Shojaee et al. have reported no significant change in the endothelial cell density of the cornea after the administration of topical MMC during PRK; the postoperative haze grade was notably lower in the MMC group (7).

Light microscopic examination of the exenteration material from two patients with conjunctival melanoma who underwent 0.4 mg/mL topical MMC treatment for 7 and 28 days subsequently did not reveal toxic effects on the episclera, cornea, iris, ciliary body, lens or retina (8). However, the findings of that study were limited by a short-term application of MMC and evaluation under light microscopy, which is not as sensitive as electron microscopy. Therefore, minimal changes may not be observed using this method.

Avisar and co-workers applied 0.2 mg/mL topical MMC for 5 min during pterygium surgery (9). Preoperative endothelial cell count of 2254 ± 128 cell/ mm^2 decreased to % 21.25 ± 2.8 at the postoperative first week, %24.26 ± 1.8 at the first month and % 21.05 ± 3.2 at the third month. Consequently, the difference in the values was significant at all times.

We evaluated the effects of MMC on the cornea under light microscopy with hematoxylin and eosin and observed a significant linear increase in epithelial cell numbers in groups who received a high dose numbers in groups who received a high dose MMC for a long period of time (0.4 vs 0.8 mg/mL). These findings were not prominent at lower doses. Compared with the control group, the severity of vacuolar degeneration and, cellular disarrangement in the corneal endothelial cells of the treatment group was correlated to a high dose and short administration period. Electron microscopic examination of the epithelium revealed enlargement of intercellular areas due to edema, presence of electrodense cells, and decrease in intercellular binding complexes. The following results were also observed: increases in nuclear chromatin, profound indentations, expansion of mitochondria, and cytoplasmic vacuolization. The stroma was normal except for expansion in fibroblasts. Thinning of the endothelial cells, loss of superficial microvilli and cytoplasmic vacuolization were common findings. The severity of these findings was also in accordance with the dose and administration period of MMC.

The cornea of rabbit differs from that of humans,i.e., the former does not have the Bowman membrane; thus, it is thinner.

In addition, the corneal endothelial cells of rabbits reproduce via mitosis. Therefore, the results obtained in rabbits can not be generalized to humans.

MMC is markedly effective on proliferative cells. However, the cornea of rabbits is more sensitive because it is thin and exhibits mitotic activity in the endothelium. The risk of endothelial failure is extremely low because the damaged endothelium can be repaired by mitosis (3,10).

Due to the antineoplastic preperties of MMC, it is widely used for tumor therapyand for surgical interventions as an antiproliferative agent. However, severe and irreversible side effects may develop after, the application of MMC soaked sponges in glaucoma and pterygium surgery. In individual with ocular surface tumors, long-term administration of MMC can cause side effects. Therefore, studies on agents with antineoplastic vs antifibrotic effects are ongoing. Among them, the most important studies are those on fluorouracil and interferon-2b. The adverse effects of these agents seem to be less than those of MMC; however, more studies must be conducted (11).

In our study, long-term administration of MMC at low doses produced cytotoxic effects in the cornea. However, these findings were severe in groups who received high doses (0,8 mg/mL). Short-term high dose treatment resulted in severe histopathologic changes in the cornea compared with long-term low-dose treatment. Therefore, the most effective low dose of topical MMC should be administered to prevent irritation and possible cytotoxic effects. This will prevent, the loss of limbal stem cells, punctal stenosis, ocular irritation, conjunctival hyperemia, lacrimation, punctate keratopathy, blepharospasm, corneal edema and ocular pain due to topical MMC-administration. Several studies have assessed various doses and methods. Treatment with low-dose MMC (0.02 mg/mL) for primary and reccurrent ocular surface tumors was found the result in tumor regression without any side effects (12).

Currently, MMC is widely used in ophthalmology, and its side effects on the cornea because of high-dose treatments are well known. Regarding side effects, long term low-dose treatment with MMC may be more advantageous than short-term high-dose treatment; therefore, the former should be preferred. MMC is also being used in refractive surgery, where these side effects have been observed. In non-regressed ocular surface tumors, the num-

ber of cures should be increased. Furhermore, a decrease in the current concentration will facilitate better tolerence of the drug.

It remains unclear whether histopathologic changes in the cornea due to MMC treatment are reversible or permanent. We did not evaluate histopathologic changes after the termination of the treatment, however, studies should be conducted to address this issue and to clarify the safe and effective MMC dose and treatment duration. There is not consensus regarding MMC dose and treatment duration,as currently, there is no consensus regarding these. Because of the severe side effects of treatment with topical MMC, surgery appears to be the first-line treatment for ocular surface tumors. Therefore, MMC is administered in inactive doses, or it is discontinued early. This study aimed to determine the adequate application period for the lowest active dose and the occurrence of side effects in these circumstances. We evaluated the histopathologic changes in the cornea caused by treatment with topical MMC at various doses and application periods. Significant changes in the cornea wereobserved in the high- dose MMC treatment groups.

CONCLUSION

In terms of side effects, long-term low dose treatment with topical MMC than short-term high-dose treatment; therefore, the former should be recommended.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Animal Ethics Review Committee of the Çukurova University School of Medicine.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors have no conflicts of interest to declare.

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Original Research

Dynamic Thiol/ Disulfide Balance in Children with Acute Malnutrition

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ABSTRACT

Objective: There is a balance among the free radical production and the antioxidant system suppressing the increase of reactive oxygen species in the body. If this balance is disturbed, then oxidative stress occurs. Free radicals caused by normal metabolism or pathological processes cause deterioration in the structure and functions of thiol-dependent enzymes and change in the thiol / disulfide balance in the cell environment. The aim of our study is to evaluate the dynamic thiol/ disulfide balance in children with acute malnutrition.

Methods: The weight, weight Z score, height, height Z score and BMI of the patients in the study group were measured. 52 patients diagnosed with acute malnutrition according to Waterlow classification and 40 healthy children were included in the study. The thiol/ disulfide balance was measured in both groups by using the automatic method developed by Erel and Neselioğlu.

Results: There was no statistically significant difference among patient and control group in terms of the average native thiol and total thiol levels. While it was found that the levels of disulfide, disulfide/native thiol and disulfide/total thiol were statistically significant in the patient group, the native thiol/total thiol ratio was found to be lower at a significant level.

Conclusion: It was seen that thiol/ disulfide balance weakened in children with acute malnutrition and showed a shift towards disulfide.

Keywords: Children, malnutrition, oxidative stress, thiol/ disulfide balance.

INTRODUCTION

Malnutrition is a clinical case arising as a result of inadequate intake of one or more food items to disrupt the body balance (1). Malnutrition is defined as the fact that normal body weight by age, height by age and/or body weight by height is below -2 SD due to lack of protein and/or energy. Malnutrition is a major health problems in underdeveloped and developing countries, forming an important part of the world's population (2). In our country, it continues to be an important health problem affecting the pediatric age group. Although it is easy to diagnose patients with severe malnutrition; it is very difficult to diagnose patients with mild, moderate malnutrition. It is very important to detect mild and moderate malnutrition and prevent chronic and severe malnutrition. In these cases, various methods have been tried to be developed for early diagnosis (3).

Different markers are used to evaluate the oxidant / antioxidant balance (4, 5). Thiol / disulfid balance is a new method developed by Erel et al. (6) which is used in the evaluation of oxidant / antioxidant balance. Thiol balances oxidative stress by reducing reactive oxygen species or by accelerating inactivation. The reactive oxygen species and the thiol groups present in the environment are oxidized and transformed into reversible disulfide bonds. The disulfide is oxidized by oxidizing molecules in the environment and converted into reversible bond structures. The resulting disulfide bond structures can be reduced back to the thiol groups, thereby maintaining the thiol disulfide balance. The thiol disulfid balance has a critical role in antioxidant defense, detoxification, apoptosis, regulation of enzyme activities, and mechanisms of transcription and cellular signal transduction (7, 8).

Purpose of the study is to investigate thiol/ disulfide balance effect, a recently discovered indicator of oxidative stress triggered by slowing and increasing catabolic processes in basal metabolic processes of patients with malnutrition, on acute malnutrition patients.

METHODS

In this study, children who were admitted to the general pediatric outpatient clinic with the complaint of developmental retardation and whose weight was below 3% percentile were included in the study. Percentage charts developed by Olcay Neyzi et al. (9) were used for weight and height measurements. The weight,

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weight Z score, height, height Z score and BMI (Body Mass Index) of the patients were calculated. Waterlow classification was used for the malnutrition criterion (10) (Table 1). A detailed story of all the children included in the study was received and detailed physical examinations and laboratory examinations were carried out. The approval of ethical committee was received. Informed consent was obtained from the parents of all cases included in the study.

Exclusion criteria

Children with acute infections, tuberculosis, congenital anormalities, epilepsy, mental motor retardation, diabetes mellitus, hypothyroidism, celiac disease, acute and chronic diseases of organs such as kidney, lung, liver and heart were not taken in the study.

Blood samples

Before starting the study, complete blood count of patients and children in the control group were done with automatic blood count device (Abbot Cell dyn 3500 lll, USA). Blood samples taken from the selected cases for the study were centrifuged at 3500 rpm for 10 minutes and then shaped elements were thrown away together with the tube. Some of the serum samples at the top were stored at -80 ^{0}C. On the same day, with the remaining serum samples electrolytes, kidney and liver function tests, TSH, free T4, tissue transglutaminase IgA, endomysium IgA, CRP, vit D level, B12, folic acid were studied on whereas the serum stored at -80 ^{0} C was used to detect dynamic kinetic thiol/ disulfide levels on the day studied.

Table 1. Waterlow classification used in malnutrition

	Waterlow Classification	
Degree of malnutrition	**Wasting(%) Weight for height**	**Stunting(%) Height for age**
Normal: Grade 0	90–110	>95
Mild: Grade 1	80–89	90–94
Moderate: Grade 2	70–79	85–89
Severe: Grade 3	<70	<85

Measurement of thiol / disulfide balance parameters

The thiol/ disulfide balance tests were measured using a newly developed Erel&Neselioglu's novel technique (6). The serum thiol/ disulfide balance parameters were measured Roche Hitachi Cobas c501 automatic analyzer. The amount of disulfide was calculated with the formula of (total thiols-native thiols)/2. After the native (SH) and total thiols (-SH + –S – S–) were determined, the disulfide levels (SS), reduced thiol ratio (native thiol / total thiol), oxidized thiol ratio (disulfide/total thiol) and thiol oxidation-reduction ratio (disulfide / native thiol) other parameters are obtained by mathematical calculation (6).

Statistical Analysis

We performed the statistical analyses using NCSS (Number Cruncher Statistical System) 2007 program (Kaysville, Utah, USA). When evaluating the study data, the differences among the groups were examined using the Student t-test and Pearson-chi square test. Spearman's Correlation Analysis was used in evaluating correlations. The limit value for statistically significant differences was 0.05.

RESULTS

In our study, 24 of the 52 patients with acute malnutrition were male (46.2%) and 28 were female (53.8%). The mean age in the patient group was 88.54±55.71 months. From the 40 patients, taken in the study as control group, 21 were male (%52.5) and 19 were female (%47.5). The mean age of the control group was 75.15 ± 49.50 months. No statistically significant difference was detected among the two groups in terms of age and gender (p>0.05) (Table 2).

While there was no statistically significant difference among the two groups in length measurements (p>0.05), there was statistically significant difference among height Z score, weight, weight Z score and BMI ratios (p< 0.05) (Table 2).

From the perspective of thiol/ disulfide balance, the average native thiol levels in patients and control groups were determined as 388.48±59.74 and 407.03±61.20 mmol/l, respectively. There was no statistically significant difference found in terms of average

Table 2. Demographic characteristics of the study population.

	Acute malnutrition (n=52)	Control (n=40)	p
Age (month)	88,54±55,71	75,15±49,50	0,234
Gender; n (%)			
Male	24 (46,2)	21 (52,5)	0,546
Female	28 (53,8)	19 (47,5)	
Weight	18,07±9,27	24,30±12,96	0,012*
Weight Z score	–1,98±0,51	0,35±0,48	0,001*
Height (cm)	110,40±24,89	115,65±23,99	0,340
Height Z score	–1,95±0,69	0,68±0,73	0,001*
BMI (kg/m^2)	13,91±1,57	17,00±1,95	0,001*

Note. Parameters are expressed as means ± standard deviation, BMI: Body mass index;
*p< 0.05 was considered statistically significant.

Table 3. Thiol/ disulfide balance parameter levels among groups.

	Acute malnutrition	Control	p
Native thiol [µmol/L]	388,48±59,74	407,03±61,20	0,140
Total thiol [µmol/L]	461,42±60,21	444,05±61,74	0,190
Disulfide [µmol/L]	36,47±18,08	18,51±7,31	0,001*
Disulfide/Native thiol (%)	9,92±5,89	4,66±2,01	0,001*
Disulfide/ Total thiol (%)	7,93±3,67	4,20±1,66	0,001*
Native thiol/Total thiol (%)	84,15±7,33	91,59±3,33	0,001*

Note. Parameters are expressed as means ± standard deviation.
*p < 0.05 was considered statistically significant

Table 4. The correlation analysis of thiol/ disulfide balance parameters and other risk factors in the acute malnutrition.

		Native thiol [µmol/L]	Total thiol [µmol/L]	Disulfide [µmol/L]	Disulfide/ Native thiol (%)	Disulfide/ Total thiol (%)	Native thiol/ Total thiol (%)
Age (month)	r	0,169	0,115	-0,040	-0,075	-0,075	0,075
	p	0,230	0,419	0,779	0,598	0,598	0,598
Weight	r	0,161	0,099	-0,045	-0,076	-0,076	0,076
	p	0,254	0,484	0,752	0,590	0,590	0,590
Weight Z score	r	-0,151	-0,162	0,039	0,069	0,069	-0,069
	p	0,284	0,252	0,786	0,628	0,628	0,628
Height (cm)	r	0,148	0,095	-0,030	-0,061	-0,061	0,061
	p	0,294	0,505	0,832	0,665	0,665	0,665
Height Z score	r	-0,006	0,098	0,141	0,115	0,115	-0,115
	p	0,966	0,491	0,317	0,418	0,418	0,418
BMI (kg/m^2)	r	-0,091	-0,107	0,000	0,028	0,028	-0,028
	p	0,521	0,452	0,998	0,844	0,844	0,844

Note. BMI: body mass index;
*p < 0.05 was considered statistically significant.

native thiole levels (p =0,140; p>0,05). Total thiol levels were determined as 461.42 ± 60.21 and 444.05 ± 61.74 mmol/l respectively in patient and control groups; there was no statistically significant difference among the two groups (p=0,190; p>0,05). However, with regards to disulfide levels among the two groups, disulfide levels in the patient group (average 36.47 ± 18.08) were found statistically higher in a significant way than in the control group (average 18,51±7,31) (p=0,001; p<0,05). While the ratio of disulfide/ native thiol and disulfide/total thiol in the patient group were detected significantly higher in the patient group than in the control group (p=0,001; p<0,05) (p=0,049; p<0,05), native thiol/total thiol ratios in the patient group were found statistically lower in a significant way than in the control group (p=0.001; p<0.01). The thiol/ disulfide balance parameters of both groups are shown in table 3. Patient group thiol/ disulfide balance tests and correlation analysis among other parameters are shown in Table 4 in detail.

DISCUSSION

Malnutrition is a pathological condition arising as a result of deficient or unbalanced intake of one or more food items. This situation can be due to protein deficiency, energy deficiency, or both (11).

Free oxygen radicals, synthesized in the body in a small amount and do not harm the body during normal metabolism, are produced in excessive quantities in viral diseases, in some situations such as exposure to ionizing radiation and environmental pollution and result in oxidative stress (12).

There is a balance among the free radical production and the antioxidant system suppressing the increase of reactive oxygen species in the body. If this balance is disturbed, then oxidative stress occurs. In previous studies on oxidative stress; enzymatic antioxidant levels such as malondialdehyde, glutathione peroxidase and catalase were measured (12). In the studies, it was found that serum oxidative stress level was higher in patients with malnutrition compared to the control group and antioxidant capacity was found to be low (13). In another study, both total serum oxidative capacity and total antioxidant capacity were lower in malnourished patients than in the control group

(14). It was thought that this may be related to the decrease in endogenous free radical production due to body metabolic processes and slowing of energy consumption in malnutrition (14). Recently, the plasma thiol / disulfide balance measured by a new method developed by Erel and Neselioglu (6) is used to obtain information about oxidative stress. We also used this method, developed in 2014 and measuring the thiol/ disulfide homeostasis, in our study.

Thiol groups both separate antioxidant and regulate the redox system. Dynamic thiol/ disulfide balance has critical roles in antioxidant protection, detoxification, signal transmission, apoptosis, enzyme activity and regulation of cellular signal mechanism by transcription factor (6, 7, 8, 15). During abnormal thiol/ disulfide balance, these vital cellular functions deteriorate. Due to oxidative stress, pathologies occur in organelles such as mitochondrial, vesicles and cell membrane resulting in imbalances. Also inflammation, chemistry and oxidant radicals generated by other factors such as radiation may damage the thiol/ disulfide balance (6, 16). In both cases described above, thiol/ disulfide balance is expected to be weakened compared to the control group.

Until present day, studies on the role of dynamic thiol/ disulfide balance have been made in the etiopathogenesis of many diseases. In a study conducted on vitiligo patients by Üstüner (17) total thiol levels were determined to be higher than the control group. It was stated that disulfide measurements, disulfide/native thiol and disulfide/total thiol ratios of vitiligo patients were higher than the control group. It was also stated that the serum total thiol and disulfide levels correlated with disease activity in vitiligo patients and that this can be used as a new inflammatory indicator in determining the prognosis of the disease. Again in a study, conducted on autoimmune subclinical hypothyroid patients by Ateş et al. (18) native thiol levels were lower in the patient group, although not statistically significant, total thiol levels were also low. Disulfide and disulfide/native thiol and disulfide/total thiol were reported to be higher and balance was reported to shift in favor of disulfide.

In the field of pediatrics in the literature there are very few studies conducted on the role of thiol/ disulfide balance. In the study done by Durmuş et al. (19) in children with type 1 diabetes mellitus (T1DM), disulfide/native thiol and disulfide/total thiol ratios were significantly higher whereas the native thiol and native thiol/total thiol levels were lower in the patient group. disulfide/native thiol and disulfide/total thiol were reported to be higher and balance was reported to shift in favor of disulfide. Again in a study, conducted on pediatric adenoid hypertrophy patients by Ozdamar et al. (20) disulfide, disulfide/native thiol and disulfide/total thiol values were found to be higher. It was found that the native thiol and total thiol values in these patients were lower than those of the healthy group. Disulfide and disulfide/native thiol and disulfide/total thiol were reported to be higher and balance was reported to shift in favor of disulfide. Again in the study, conducted on children with duchenne muscular dystrophy patients by Incecik et al. (21) total thiol level and native thiol levels were lower in the patient group, although not statistically significant, the disulfide, disulfide/native thiol, and disulfide/total thiol ratio were also low.

In our study, we evaluated thiol/ disulfide balance, in acute malnutrition patients. There was no statistically significant difference among the two groups when the native thiol and total thiol levels were evaluated. While disulfide level, disulfide/native thiol ratio, and disulfide/total thiol ratio in the patient group were detected significantly higher, native thiol/total thiol ratios in the patient group were found statistically lower in a significant way than in the healthy group. In our study, we found that in children with acute malnutrition, the thiol/ disulfide balance shifted to the disulfide side, ie to the right, indicating increased oxidative stress. Increased disulfide, disulfide/total thiol (oxidized thiol ratio) and disulfide/native thiol (thiol oxidation-reduction ratio) ratio show increased oxidative stress, whereas low native thiol / total thiol ratio in children with acute malnutrition compared to healthy children also indicates low antioxidant defense mechanism. This is due to the increase in free radicals and oxidative stress caused by the suppression of immune systems in patients with malnutrition and the low intake of foods such as protein, glucose and vitamins. To the extent we know, our study is the first to examine the dynamic thiol / disulfide homeostasis in children with acute malnutrition in the literature.

As stated above, there is evidence that abnormal thiol/ disulfide balance have a role in the pathogenesis of some diseases. Therefore, detection of dynamic thiol/ disulfide balance can provide valuable information about a variety of normal or abnormal biochemical processes.

In our study, there was no relationship found among the age, gender, weight, weight Z score, height, height Z score and BMI measurements of sick children and thiol/ disulfide balance tests. However, in the study carried out by Ateş et al. (18), there was negative correlation found among age of autoimmune subclinical hypothyroid patients and native thiol level, total thiol level, disulfide/native thiol ratio and disulfide/total thiol ratio; also showed a positive correlation with disulfide level and the ratio of natural thiol/total thiol. It was also stated that the level of disulfide increases with the oxidation of thiols as the level of obesity increases (6). Also, it showed that as age increases, disulfide levels increase, thiol levels decrease. In addition, in previous studies it has been reported that oxidative stress increases with age and that thiol/ disulfide homeostatic imbalance occurs as a result of this (6).

Limitations and Strengths

In our study, it is difficult to generalize the results of all patients to acute malnutrition because of the low number of patients. Furthermore, our study did not take the evaluation of thiol/ disulfide balance parameters according to the degree of acute malnutrition patients. In our study, dynamic thiol/ disulfide balance, which is only part of the complex mechanism of oxidative stress, was evaluated, while other oxidative stress parameters were not evaluated. We could not determine the relationship among dynamic thiol/ disulfide balance, other oxidative stress parameters and acute malnutrition. Thiol disulfide balance, in

different large patient groups according to the degree of acute malnutrition, other oxidative stress parameters or new biomarkers should be examined in future studies to further validate our findings. On the other hand, the main strength of our study is that all the thiol/ disulfide parameters were measured with a new and fully automated method. And also the strict exclusion criteria were applied in order to minimize the confounding variables.

CONCLUSION

As a result, in our study of thiol/ disulfide balance in patients with acute malnutrition, we have found that this balance was weakened in children with acute malnutrition and showed a shift towards disulfide. It was observed that children with acute malnutrition increased total oxidative stress activity and total antioxidant level decreased compared to the healty group. There are no studies in the literature in which thiol/ disulfide balance in children with acute malnutrition is evaluated. In the matter of supporting our data, we believe that there is a need for studies with more patients in this matter.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Harran University Faculty of Medicine Ethics Committee (11.05.2017/05).

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Original Research

The Relationship Between Cd 74 Levels, Macrophage Migration Inhibitory Factor Gene Polymorphism and Clinical Features in Patients with Ankylosing Spondylitis

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ABSTRACT

Objective: In this study, the primary objective was to compare CD 74 antigen levels between patients with ankylosing spondylitis (AS) and healthy controls. The secondary objective was to investigate the distribution of Macrophage Migration Inhibitory Factor (MIF) 173 G/C polymorphisms in AS patients and a control group. Finally, it was also aimed to reveal the presence of a relationship between CD 74 antigen levels and MIF 173 G/C polymorphism.

Materials and Methods: 82 healthy blood donors and 79 AS patients were enrolled in this study. MIF 173 G/C polymorphism and CD 74 levels were investigated in the patient and control groups using the ELISA method. Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Radiology Index (BASRI), Visual Analogue Scale (VAS) and Ankylosing Spondylitis Quality of Life (ASQoL) scores were calculated and recorded.

Results: There was no significant difference between the patient and control groups in terms of age, gender, and body mass index. The median CD74 level of the patient group was 1.17 (0.93-2.1), which was significantly lower than that of the control group, i.e. 2.16 (1.6-4.41). There was no correlation between CD 74 antigen levels and BASDAI, BASMI, BASFI, BASRI, VAS and ASQoL scores. The number of patients who had the C allele was higher among the patients with AS in comparison to the control group; however, the difference was not statistically significant ($p>0.05$). There was no correlation between genotypes and BASDAI, BASMI, BASFI, BASRI, VAS and ASQoL scores ($p>0.05$). Comparison of the median CD74 levels of the individuals in the patient group according to their HLA-B27 status and genotypes did not reveal any statistically significant difference.

Conclusion: The CD74 antigen levels of the patients with AS were significantly lower compared to the control group. This implies that CD 74 is a parameter that can be used in the diagnosis of AS. Although the C allele among the MIF 173 G/C polymorphisms was more frequently observed in the patient group, the difference was not statistically significant. Moreover, there was no significant relationship between CD 74 antigen levels and polymorphisms.

Keywords: Ankylosing Spondylitis, CD 74, MIF gene, Polymorphism

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic and progressive inflammatory disease characterized by axial skeletal and sacroiliac joint involvement (1). AS is the most prevalent type of spondyloarthropathies (SpA). While it can cause enthesitis and peripheral joint involvement, it can also exhibit extraarticular involvement (2). AS onset is generally seen during late adolescence and early adulthood. One of the most important characteristics of the disease is axial involvement wherein nearly 90% of the patients exhibit radiographic sacroiliitis as the disease progresses. The diagnosis can be delayed since it can take years to observe the radiographical signs although the clinical signs of the disease are observed earlier (3).

The etiology of AS is not entirely known. However, it is thought to stem from environmental factors connected with genetic fac-

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tors (4). Among the genetic factors, HLA-B27 (Human Leukocyte Antigen) is used to help diagnose AS. On the other hand, new biomarkers are needed for the diagnosis, follow-up and determination of the prognosis, since a minority of the HLA B-27-positive individuals develop AS and healthy individuals can also be HLA-B27-positive (5). From the genetic viewpoint, besides HLA-B27 AS was also found to be associated with Endoplasmic Reticulum Aminopeptidase I (ERAP1) and Tumor Necrosis Factor Alpha (TNF-α) genes (6,7).

The CD 74 antigen, which is a transmembrane glycoprotein, prevents the early binding of proteins to the MHC class II (Major Histocompatibility Complex). A CD 74 molecule has intracellular regulatory functions such as signal transduction, cell migration and endosomal trafficking (8, 9). It also plays a role in innate and acquired immunity and B cell proliferation. Pro-inflammatory cytokines are released as a result of antibody binding to the CD 74 antigen (8). Therefore, it is thought that CD 74 can be a potential factor in the etiopathogenesis of AS.

Macrophage Migration Inhibitory Factor (MIF) is a multifunctioning mediator protein that consists of 115 amino acids with enzyme, hormone and cytokine properties that can inhibit macrophage migration in vivo (10). After it is secreted extracellularly, it promotes pro-inflammatory activity and increases TNF release by affecting the immune response via autocrine, paracrine and endocrine routes. MIF also acts as a regulator against the immunosuppressive effects of glucocorticoids and hence, it was thought that MIF could be a gene associated with autoimmune-inflammatory diseases (11). It has been demonstrated that MIF is able to reverse the effects of glucocorticoids on inflammation in an antigen-related arthritis model (12). CATT tetra-nucleotide polymorphisms and 173 G/C polymorphism of the MIF gene were found to be associated with inflammation (13,14).

The CD 74 antigen located on the cell surface acts as a receptor for MIF. CD 74 and MIF binding plays an important role in maintaining cell proliferation and cell viability (15).

In designing this study, the primary objective was to determine whether CD 74 antigen levels measured with the ELISA method could be used as a parameter in the diagnosis, treatment and follow-up of AS. The secondary objective was to investigate the role of 173 G/C polymorphism of the MIF gene in the development of AS. Furthermore, we also aimed to reveal the presence of a relationship between CD 74 antigen levels and MIF gene polymorphisms.

MATERIALS AND METHODS

82 healthy subjects and 79 patients diagnosed with ankylosing spondylitis (AS) who presented at Medical Faculty Research Hospital, Physical Medicine and Rehabilitation Department/Rheumatology outpatient clinic between December 2016-April 2017 were enrolled in this study. Patients with ankylosing spondylitis were selected according to the Modified New York criteria. Blood donors who did not have any chronic diseases and/or history of inflammatory disease were recruited as the control group. Patients with an inflammatory disease besides AS, pregnant or breast-feeding women, and those with a history of malignancy were not included in the patient group. Consent was obtained from all individuals included in the study before participating in the study. The study was approved by University Ethics Committee (28.11.2016-308) and the study was performed according to the Declaration of Helsinki.

The patients were asked about their age, gender, height, weight, date of onset of complaints associated with the disease, duration of the disease, involvements, drugs prescribed, family history and extraarticular involvement. The same physician performed the general physical examination and the detailed musculoskeletal examination of all patients. In the patient group, disease activity was evaluated using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), metrologic evaluation was performed using Bath Ankylosing Spondylitis Metrology Index (BASMI), functional evaluation using Bath Ankylosing Spondylitis Functional Index (BASFI), radiologic changes using Bath Ankylosing Spondylitis Radiology Index (BASRI), nighttime and daytime pain status using Visual Analogue Scale (VAS) and quality of life using Ankylosing Spondylitis Quality of Life (AS-QoL) scale.

Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) values were assayed from blood samples of the patients on the same day and recorded.

Genomic DNA was extracted using the salting out method from mononuclear cells obtained from peripheral venous blood treated with ethylenediaminetetraacetic acid. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to evaluate MIF gene polymorphisms. MIF-173C *rs755622 variant was identified using the following primers: 5'ACTAAGAAAGACCCGAGG-3 ', 5'-TGGAGAAAGGACCAGGAGAC-3'. MIF-173 G/C sequences were amplified to a total reaction volume of 25 µl using 100-500 ng DNA, 1.0 mM of each primer, 250 mM of each nucleotide, 1.5 U Taq polymerase, (Fermentas International, Burlington, Ontario, Canada), and 2 ml 10x PCR buffer. PCR was performed using GeneAmp® PCR System 9700 (Applied Biosystems, Singapore).

A 330 bp fragment was amplified for MIF-173 G/C, and then digested with Alu I restriction endonuclease (Invitrogen, Carlsbad, CA, USA) at 37°C overnight. Digestion products were dissolved in 3% agarose gel and viewed under UV light. 330 bp PCR products have a consistent restriction region that leads to 62 and 268 bp fragments. GG genotype did not have a second cutting point for Alu I. CC genotype had a second cutting point and led to three fragments, i.e. 205, 62 and 63 bp fragments. The experiment was repeated for 20% of the samples in order to prevent sampling or reading errors.

The CD 74 concentration was measured using Elabscience brand ELISA kits. The measurement was carried out in accordance with the user manual in the kit. The microplates in the kit were spectrophotometrically read at 450 nm with an EL 312 Microplate brand ELISA reader. The obtained OD (Optical Density) values were used to create standard curves and concentration values were obtained in ng/ml.

Table 1. Comparison of the demographic characteristics between the patient group and the control group

		Group		
		Patient (n=79)	**Control (n=82)**	**p**
Gender	Male	64 (81.01)	73 (89.02)	0.154*
	Female	15 (18.99)	9 (10.98)	
Age		36.01±9.14	36.63±10.31	0.687**
BMI		27.95±4.61	26.76±4.09	0.084**

* : Chi–square test was used. Descriptive statistics were provided in numbers (%).
**: Independent samples t test was used. Descriptive statistics were expressed as mean±standard deviation.

Table 2. Comparison of CD 74 antigen levels and polymorphisms between the patient group and the control group

		Group		
		Patient (n=79)	**Control (n=82)**	**p**
CD74		1.17 (0.93–2.1)	2.16 (1.6–4.41)	p<0.001**
Genotype	GG	56 (70.89)	62 (75.61)	0.581*
	GC	20 (25.32)	19 (23.17)	
	CC	3 (3.8)	1 (1.22)	

* : Chi–square test was used. Descriptive statistics were provided in numbers (%).
**: Mann Whitney U test was used. Descriptive statistics were expressed as median values (Q1–Q3).

Table 3. Comparison of CD 74 antigen levels according to HLA–B27 and polymorphism status in the patient group

	CD 74	**M(Q1–Q3)**	**p**
HLA–B27	HLA–B27+ (n=49)	1.39 (0.89–2.27)	0.377*
	HLA–B27– (n=30)	1.08 (0.97–1.48)	
Genotype	GG (n=56)	1.17 (0.9–2.06)	0.077**
	GC (n=20)	1.1 (0.93–1.855)	
	CC (n=3)	5.24 (1.77–11.82)	

* : Mann Whitney U test was used. Descriptive statistics were expressed as median values (Q1–Q3).
**: Kruskal Wallis test was used. Descriptive statistics were expressed as median values (Q1–Q3).

Statistical Analysis

Normal distribution of numerical variables was tested using the *Shapiro Wilk test* when n<50, and the *Kolmogorov Smirnov* test when n>50. Independent *Samples t-test*, which is a parametric test, was used in comparing two independent groups when numerical variables had a normal distribution, and the *Mann Whitney U test* was used in the case of non-normal distribution. The *Kruskal Wallis test* was used to compare more than two independent groups when numerical variables did not have a normal distribution. In non-parametric tests, the differences between the two groups were compared using the *Mann Whitney U test* and evaluated using the *Bonferroni correction*. The Pearson Chi-Square test was used to compare the differences between categorical variables in 2x2 tables, and the *Fisher Freeman Halton Test* in RxC tables. Relationships between the numerical variables that did not have a normal distribution were evaluated using the *Spearman Rho Correlation Coefficient*. Statistical analyses were performed using R 3.3.2 v (open source) software and 0.05 (p-value) was used as the level of significance in statistical analyses.

RESULTS

79 patients and 82 healthy subjects were enrolled in this study. Age, gender and body mass index distributions were similar in the patient and control groups (p>0.05 for each) (Table 1).

The mean time until diagnosis was 6.87±5.55 years in the patient group. Of the 79 patients included in the study, 29 were on Disease Modifying Drugs (DMARD) and 50 were receiving anti-TNF therapy.

The median CD74 level of the patient group was 1.17 (0.93-2.1), which was significantly lower than that of the control group, i.e. 2.16 (1.6-4.41) (p<0.001). The prevalence of C allele presence was higher in the patient group (29.12%) as compared to the control group (24.39%); however, the difference was not statistically significant (p>0.05) (Table 2).

CRP scores increased in parallel to the CD 74 levels in the patient group (p=0.015). Comparison of the CD 74 scores with BMI, time until diagnosis, ESR, nighttime VAS, daytime VAS, basdai, basmi, basri vertebra, basri hip, basri total and AS-QoL in the patient group did not reveal a significant linear relationship (p>0.05 for each).

49 individuals (62%) in the patient group were HLA-B27-positive, whereas 30 individuals (38%) were HLA-B27-negative. Comparison of the median CD74 levels of the individuals in the patient group according to their HLA-B27 status and genotypes did not reveal any statistically significant difference (p>0.05 for each) (Table 3).

Comparison of the CD74 levels of the individuals in the patient group according the drugs prescribed, presence or absence of arthritis, uveitis and enthesitis did not reveal any statistically significant difference in terms of the median values ($p>0.05$ for each). There was no statistically significant relationship between the genotypes and the clinical parameters of the subjects in the patient group ($p>0.05$ for each).

DISCUSSION

AS is a chronic and progressive inflammatory disease that initially manifests with axial skeletal and sacroiliac joint involvement, and its etiology is not entirely known. Delayed diagnosis is one of the most important problems in AS. The diagnosis can be delayed for 7 to 10 years, since there is no test that can directly establish a diagnosis of AS. Therefore, new biomarkers are required for the diagnosis, follow-up and estimation of the prognosis of AS.

It is thought that many different factors may play a role in the etiology of AS together with its genetic causes. A minority of HLA-B27-positive patients develop AS. Therefore, it is asserted that HLA-B27 can only partly account for a genetic susceptibility, although it is the most commonly recognized genetic factor for the disease (16).

MIF is a molecule that plays a role in the regulation of innate and acquired immunity as well as carcinogenesis and inflammation (17, 18). Suppression of the biological activity of MIF can significantly limit TNF-α, interferon-γ and matrix metalloproteinase production in the large intestinal tissues in an experimentally induced murine colitis model (19). The MIF gene and its polymorphisms in inflammatory diseases have been widely investigated due to the close relationship between MIF and inflammation and its role as a regulator against glucocorticoids.

MIF gene polymorphism was most commonly investigated in Rheumatoid Arthritis (RA). It was found that MIF gene polymorphism resulted in a susceptibility to the disease and was correlated with radiological progression in patients with RA. Martinez et al. found that the MIF-173 C allele in the promotor was especially associated with a susceptibility in early-onset RA patients (20). In another study, it was reported that the MIF-173 C allele or MIF CATT alleles were correlated with serum MIF levels and they could be used as prognostic factors (21).

Zheng et al. investigated MIF levels as well as MIF-173 G/C and -794 CATT polymorphism in 600 healthy subjects and 600 patients with Behçet's disease. They found that the prevalence of the C allele was higher in the patient group compared to the control group. They also found that MIF levels were higher in the patient group compared to the control group and that polymorphism could have an effect on MIF expression (22).

Przybyłowska et al. investigated MIF gene polymorphism in inflammatory bowel disease (IBD) in their study conducted on 58 ulcerative colitis patients, 41 patients with Crohn's disease and 436 healthy subjects. They found that the IBD risk was 2.02- and 1.89-fold higher in the presence of the G/C genotype and C allele, respectively. Considering the genotype distribution according to the subgroups, they found a relationship between Ulcerative Colitis and the C allele, but no statistically significant relationship between Crohn's Disease and polymorphism (23).

Gürel et al. investigated AS and MIF-173 G/C polymorphism in the Turkish population. As a result of the study, they found that there was no relationship between AS and MIF genotype and alleles, whereas the onset of the disease was earlier in patients who had the C allele. They asserted that the C allele could lead to earlier onset of the disease (24). According to the present study, there was no significant relationship between genotype and duration of the disease, but it was found that the patients who had the C allele experienced a longer duration of the disease in comparison to the control group.

CD 74 is a critical molecule that plays a role in various processes such as cell migration, premature antigen binding, B cell maturation and continuity of cell viability wherein its association with inflammation has been clearly shown (25, 26). Antibody binding to CD74 molecules in vivo was found to cause the production of pro-inflammatory cytokines and activation in target cells (26).

Baerlecken et al. conducted a study on 216 SpA patients and 325 control patients, and evaluated the presence of antibodies against CD74. The control group consisted of 40 patients with psoriatic arthritis, 40 patients with systemic lupus erythematosus, 40 patients with HIV, 80 patients with RA and 125 blood donors, wherein the anti-CD74 antibody level was 67% in the SpA group and 6% in the control group. They asserted that antibody positivity could be used especially in the early stage of SpA and in HLA-B27-negative patients for the diagnosis of the disease with high sensitivity and specificity (8).

Baraliakos et al. conducted a study on 94 patients with axial SpA and 51 non-SpA patients (13 RA, 17 fibromyalgia, 17 degenerative spine disease, 3 psoriatic arthritis without axial involvement and 1 polymyalgia rheumatica) to investigate the prevalence of the IgG antibody against the CLIP region of CD74. They found that the prevalence of the anti-CLIP antibody was 85.1% in axial SpA patients and 7.8% in the control group. The sensitivity and specificity of anti-CLIP were 85.1% and 92.2%, respectively in axial SpA patients. In addition, anti-CLIP antibody positivity was also detected in HLA-B27-negative patients (27). Baraliakos and Baerlecken did not observe a relationship between antibody positivity, and radiological progression and disease activity in their studies. Similarly, there was no relationship between CD74 antigen levels and disease activity, radiological involvement, drug use and extraarticular involvement according to this study.

In a study by Ranganathan et al. evaluating CD74 antigen levels in AS patients, it was found that the CD74 level was statistically significantly lower in AS patients compared to the control group. Results of this study are consistent with the results of the present study. In the mentioned study, the CD 74 level was measured from monocytes obtained from peripheral blood using the flow cytometry method (28). It is suggested that this method is not suitable for routine daily use as it is more expensive and requires advanced laboratory conditions.

The present study also showed that the CD74 levels were significantly higher compared to the control group. The researchers think this can be explained by way of several mechanisms. It is thought that the N-terminal telopeptide part, i.e. the part that activates NF-Kβ, of the CD74 molecule undergoes several proteolysis processes. It is also thought that the pathway referred to as the regulated intramembrane proteolysis is more active in AS patients. Another possible mechanism could be the fact that continuous and increased MIF signal production may lead to the cleavage and consumption of the CD74 antigen (28).

Another objective of this study was to investigate the relationship between CD 74 levels and MIF gene polymorphisms due to the close association between the CD74 molecule and MIF. However, a significant difference between polymorphism distributions and CD 74 antigen levels could not be identified. Moreover, a similar comparison could not be found in the literature.

Limitations

This study had some limitations. The mean duration of symptoms was high in the patient group and the study was a cross-sectional study. The patient group consisted of individuals who were taking drugs, and a pre- and post-treatment evaluation was not performed. Another limitation of the study was the relatively low number of patients.

Finally it is contended that CD 74 antigen levels and MIF gene polymorphisms can be used in the diagnosis, follow-up and determination of the prognosis of the disease after conducting further prospective studies with a greater number of patients.

CONCLUSION

Consequently, although MIF 173 G/C polymorphism is more frequently encountered in AS patients, there was no statistically significant difference. The afore-mentioned difference can be significant in studies that may be conducted in the future with larger patient groups and on different patient populations. It is suggested that different samples should be studied since the only study that investigates MIF gene polymorphism in AS patients has been conducted on the Turkish population. It is asserted herein that CD 74 antigen levels can be used in the diagnosis and follow-up of AS, whereby the ELISA method can be used in the measurement as a suitable method in daily practice. It is also proposed that such studies can prevent possible complications and delays in diagnosis as well as contributing to the development of new treatment options.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of University (28.11.2016-308).

Informed Consent: Written informed consent was obtained from individuals who participated in this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors have no conflicts of interest to declare.

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Original Research

Baseline Hemoglobin Levels Predict All-Cause Mortality After Saphenous Vein Graft Interventions

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ABSTRACT

Objective: Reduced baseline hemoglobin level is associated with worse outcomes in many cardiovascular conditions including percutaneous interventions of native coronary arteries. However, this relationship has not been studied in saphenous vein graft disease undergoing elective percutaneous intervention.

Metods: In this study, we evaluated baseline hemoglobin levels of 105 patients undergoing saphenous vein graft intervention. The mean follow-up was 42.4±18.4 months, and the end-point of our study was all-cause mortality.

Results: Twelve patients suffered all-cause mortality, and these patients' mean baseline hemoglobin levels were 2 g/dl lower (11.7±1.0 vs. 13.7±1.5, p=0.005), and their ejection fractions were 22% lower (49.3±10,8 vs. 38.3±12,4, p=0.014) than those of the patients without all-cause mortality. A hemoglobin cut-off 12.55 g/dL discriminated all-cause mortality with an accuracy of 85% (sensitivity: 83,3%, specificity: 76.4%, p=<0.001). Survival curve demonstrated a survival benefit of twenty-one months for the patients with hemoglobin levels higher than 12.55 g/dl (cumulative survival 95.3% vs. 70%, Log-rank p-value 0.001).

Conclusion: In summary, baseline hemoglobin levels may be associated with all-cause mortality in patients undergoing elective saphenous vein graft interventions.

Keywords: All-cause mortality, baseline hemoglobin, saphenous vein graft interventions

INTRODUCTION

As previously known, the outcomes of saphenous vein graft (SVG) percutaneous coronary interventions (PCI) were worse than native coronary artery PCI (1). Some comorbid factors may also contributes to these poor outcomes. Anemia is a well known factor that increases the risk of cardiovascular events (2-3). Association of reduced baseline hemoglobin level, and worse outcomes in percutaneous coronary interventions has been showed (4-6). Some of the studies showing this association included PCI of patients with prior CABG, however in those studies the majority of lesions were native vessels, and they included both arterial and venous conduits. Therefore, there is paucity of evidence based data in the literature focusing on saphenous vein graft interventions. The aim of our study was to analyse the impact of baseline hemoglobin levels on all cause mortality in patients undergoing SVG PCI.

MATERIALS AND METHOD

We evaluated baseline hemoglobin levels of 105 patients undergoing SVG PCI at our hospital from January 2013 to December 2018. Indications for revascularization included stable angina and all variety of acute coranary syndromes. Patients who were treated with stent, and had angiographic success were included the study. Patients with missing data for Hb levels were excluded. Patients were evaluated in the study under an institutionally approved protocol (TOBB ETÜ Hospital ethics committee, 20.06.2018/006). This was a retrospective study, performed only with screening of the medical records, hence no informed consent was obtained from the patients.

The end point of the study was all-cause mortality. Survival status was assessed by telephone contacts and checked by using national Death Notification System.

Baseline characteristics of patients including patient demographics (age, gender), past medical history (diabetes mellitus, hypertension), smoking status, low density lipoprotein, left ventricular ejection fraction, hemoglobin levels, glucose and creatinine measurements, medication use (ASA, clopidogrel/ticagrelor, anticoagulants, beta-blockers, statins, diuretics), angiographic characteristics, and PCI data were identified from computer database of our institution.

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Statistical analysis

SPSS 25 (SPSS INC, Chicago, Illinois, USA) was used for statistical analysis. Categorical variables were expressed as frequencies and continuous varibles as mean ± standard deviation. Univariate Cox regression analysis was performed to assess the association of the variables with mortality and variables that had p< 0.1 in the univariate analysis were further analyzed with multivariate Cox regression model. Receiver operating characteristic (ROC) curve was used to detect the optimal cut-off point of hemoglobin to estimate all cause mortality. Youden index was utilized to identify the best cut-off for hemoglobin. Kaplan-Meier survival curves used to assess survival times and log-rank statistics were used to test survival time differences between upper and lower cut-off point of hemoglobin. A calculated difference of p<0.05 was considered to be statistically significant.

RESULTS

During mean 42,4 month follow-up, 12 out of total 105 patients suffered all-cause mortality. Cause of mortality was reinfarction in two patients, heart failure in four patients, renal insufficiency

Table 1. Baseline Characteristics of Patients According to the Mortality Status

Variables	Mortality (–) n=93	Mortality (+) n=12	P value
Age, years	65.0(8.8)	67.3±6.4	0.291
Male / Female, %	87 /13	83/17	0.661
HT, %	54	83	0.07
DM, %	38	58	0.229
Smoking, %	32	50	0.332
Glucose, mg/dL	140.5(72.7)	174.6(64.4)	0.129
Creatinine, mg/dL	1.0(0.37)	1.5(1.2)	0.174
LDL, mg/dL	119.9(42.9)	132.8(53.6)	0.462
Hemoglobin, g/dL	13.7(1.5)	11.7(1.0)	<0.001
LVEF, %	49.0(10.8)	38.3(12.4)	0.014
Medications			
ASA, %	90	50	0.873
Clopidogrel, %	87	42	0.341
Ticagrelor, %	4	8	0.292
Varfarin, %	4	0	0.760
NOAC, %	2	0	0.873
Beta–blocker, %	68	42	0.504
ACEI/ARB, %	52	17	0.259
Statin, %	61	25	0.341
Diuretics, %	15	0	0.506

Abbreviations: DM, Diabetes mellitus; HT, hypertension; LDL, low–density lipoprotein; LVEF, left ventricular ejection fraction; ASA, Acetylsalicylic acid; NOAC, non–VKA oral anticoagulants; ACEI, Angiotensin converting enzyme–inhibitor; ARB, angiotensin receptor blocker

Figure 1. ROC curve analysis according to hemoglobin cut-off 12.55.

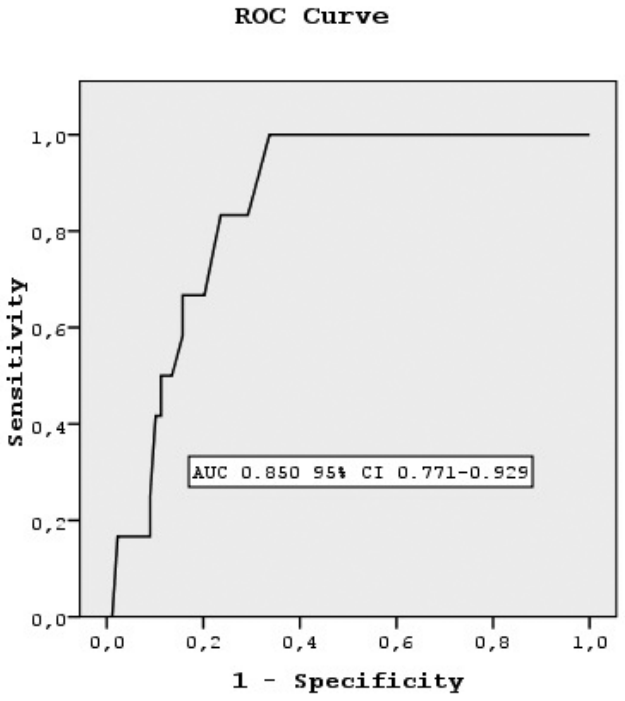
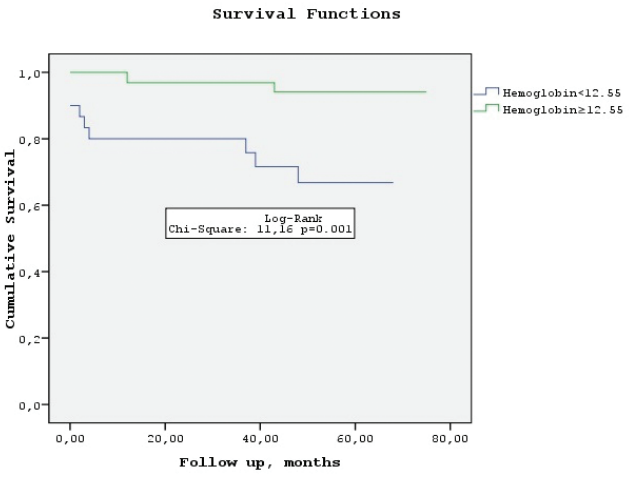


Table 2. Procedural Characteristics of Patients According to the Mortality Status

Variables	Mortality (–) n=93	Mortality (+) n=12	P value
Total Graft number	2.5(0,6)	3.0(0.7)	0.091
Treated Graft age, yr	10.2(5.8)	12.9(5.2)	0.120
TreatedGraft localization			
Diagonal	73	8	
Circumflex/OM	9	4	0.099
Right coronary/PDA	1	0	
Thrombus presence, yes	5	1	0.570
Stent diameter, mm	2.9(0.4)	3.0(0.5)	0.631
Stent length, mm	20.5(7.2)	25.2(8.0)	0.089
Stent number	1.5(0.8)	1.8(0.9)	0.320
Drug eluting stent, yes	77	10	0.591
Gp IIb/IIIa inhibitors, yes	8	1	0.683
PCI indication			
Stable angina, %	48	67	
ACS,%	52	33	0.359

Abbreviations: Gp, glycoprotein; OM, obtuse marginal branch of Circumflex coronary artery; PDA, posterior descending artery; PCI, percutaneous coronary intervention; ACS, acute coronary syndrome

Figure 2. Kaplan-Meier Survival curves according to hemoglobin cut-off 12.55.



in one patient and the others were from unknown reasons. Patients were divided into two groups according to mortality status. Baseline characteristics, and procedural characteristics are summarized in table 1 and table 2. Baseline hemoglobin levels, and LVEF were significantly different between groups (respectively 13.7±1.5 vs 11.7±1.0, p=0.005 and 49±10.8 vs 38.3±12.4, p=0.014). Cox regression analysis was performed to determine the predictors of mortality. The results of the regression analysis are shown in Table 3. Among these parameters, only hemoglobin levels were associated with mortality in multivariate analysis.

According to ROC analysis, a hemoglobin cut-off value of 12.55 g/dl discriminated all-cause mortality with an accuracy of 85% (sensitivity 83.3%, specificity: 76.4%, p<0.001) (Figure 1). There were 31 patients who had a hemoglobin value of less than 12.55 g/dl, and 74 patients had more than 12.55 g/dl in the study group. There were 9 (29.0%) mortality events in Hb<12.55 g/dl group, and 3 (4.0%) mortality events in Hb≥12.55 g/dl group (p= 0.001). During follow-up, the mortality rates according to hemoglobin cut-off value of 12.55 g/dl were estimated using Kaplan-Meier analysis (figure 2). Survival curve demonstrated a

Table 3. Predictors of Mortality after Saphenous Graft Interventions

Variables	Univariate		Multivariate	
Age, yr	1.022 (0.934–1.119)	0.638		
Male, yes	1.255 (0.151–10.440)	0.834		
HT, yes	1.919 (0.385–9.572)	0.426		
DM, yes	1.480 (0.368–5.947)	0.581		
Smoking, yes	0.953 (0.192–4.725)	0.953		
Glucose, mg/dL	1.003 (0.995–1.011)	0.462		
Creatinine, mg/dL	1.827 (1.130–2.956)	0.014	1.503 (0.788–2.869)	0.216
LDL, mg/dL	1.007 (0.992–1.022)	0.362		
Hemoglobine, g/dL	0.568 (0.373–0.863)	0.008	0.519 (0.324–0.834)	0.007
LVEF, %	0.913 (0.837–0.996)	0.041	0.958 (0.904–1.016)	0.151
Total Graft number	3.009 (0.940–9.631)	0.064		
Treated Graft age, yr	1.049 (0.932–1.182)	0.428		
Treated Graft localization		0.056		0.169
Diagonal, yes	4.106 (0.964–17.493)		3.054 (0.622–14.992)	
Other, yes	Ref.		Ref.	
Thrombus, yes	2.425 (0.292–20.151)	0.412		
Stent diameter, mm	1.434 (0.349–5.892)	0.617		
Stent length, mm	1.074 (0.987–1.167)	0.096	1.068 (0.980–1.165)	0.134
Stent number, mm	1.422 (0.721–2.804)	0.309		
Drug–eluting stent, yes	0.676 (0.135–3.376)	0.633		
Gp IIb/IIIa inhibitors, yes	1.425 (0.175–11.631)	0.741		
PCI indication	1.287(0.844–1.964)	0.241		

Abbreviations: DM, Diabetes mellitus; Gp, glycoprotein; HT, hypertension; LDL, low–density lipoprotein; LVEF, left ventricular ejection fraction; OM, obtuse marginal branch of Circumflex coronary artery; PDA, posterior descending artery

survival benefit of twenty-one months for patients with hemoglobin levels higher than 12.55 g/dl (cumulative survival 95.3% vs. 70%, log-rank p-value 0.001).

DISCUSSION

The main finding of our study is that, a low baseline hemoglobin value is independently associated with an increased all cause mortality in patients undergoing saphenous vein graft interventions. There was a progressive increase in all cause mortality as the baseline hemoglobin dropped below 13.25 g/dl.

Although coronary artery bypass surgery has been shown to increase survival, 25% of the vein grafts were occluded after five years, and 50% of them were occluded after ten years (7). Redo CABG is associated with greater morbidity and mortality, and not always with satisfactory relief of symptoms (8). Therefore a large number of patients are currently treated with PCI. PCI of SVGs has been associated with higher rates of in stent restenosis, target vessel revascularization, myocardial infarction and death compared to PCI of native vessels. Poor outcomes are related to the nature of SVG atherosclerosis, which is characterized by diffuse, concentic, friable plaques with absent or thin fibrous caps, and with high trombotic burden. Survival rates of SVG PCI is poor, and clinical factors associated with long term mortality are still elusive (9-10).

Anemia is a common comorbidity that adversely affects cardiovascular system, and associated with various cardiovascular diseases like heart failure or acute coronary syndromes. Anemia leads to a significant reduction in oxygen supply to the myocardium, and triggers myocardial ischemia in the presence of limited coronary reserve (2).

The prognostic significance of low baseline hemoglobin levels have been shown in a variety of demographic groups, and clinical settings including a diversity of cardiovascular diseases. Sabatine et al. examined baseline hemoglobin levels of about 40.000 acute coronary syndromes (ACS) patients, and they reported that patients with STEMI cardiovascular mortality of the patients increased as hemoglobin levels fell below 14g/dl, and in patients with ACS who had hemoglobin levels below 11 g/dl, had a higher mortality rate (11). Arant et al. found that hemoglobin was an independent predictor of adverse cardiovascular outcomes, with a 20% increased risk for each 1g/dl decrement in hemoglobin level in women presenting with ischemic-type symptoms (12) . Brener et al. found that baseline hemoglobin, and anemia were independent predictors of major bleeding, and death in their study consisting of 16.000 patients (13). The relation between hemoglobin levels, and mortality rate has also been shown across a broad range of indications for percutaneous coronary interventions (5,6,14). Kitai et al. demonstrated that in patients who underwent elective PCI, even mild anemia was associated with significantly higher risk for MACE (15) . Lee et al. (16), and Poludasu et al. (6) also investigated the prognostic impact of mild anemia in patients undergoing PCI. Stahli et al. found that anemia was associated with an increased all-cause mortality in patients undergoing chronic total occlusion PCI (17). Multiple mechanisims have been proposed for higher mortality in patients with anemia undergoing PCI. Increased myocardial oxygen demand (18), increased catecholamine levels, and worsening of myocardial ischemia in the presence of limited coronary reserve (2) were considered as major possible mechanisms. Procedure related blood loss, higher degrees of inflammation, co-morbidities associated with anemia were the other possible explanations for increased mortality rate.

In our study we assesed the effect of baseline hemoglobin levels on all cause mortality in PCI of SVG lesions. Similar to native vessel PCIs, baseline hemoglobin levels were found to be associated with mortality. To our knowledge, our study is the first one showing this association. Nikolsy et al included SVG PCI patients in their study, which investigated the impact of anemia on outcomes of patients undergoing PCI, but there was no subgroup analysis assessing the relation between anemia and mortality (19). The clinical factors associated with long term mortality after saphenous vein graft interventions, has been reported in a very few studies (10, 20). However, in these studies the effect of hemoglobin levels has not been assessed or demonstrated.

Patients with coronary artery bypass grafting have more extensive disease. Therefore procedural blood loss, and ischemia induced by balloon inflation during PCI may be less tolerated in the presence of anemia. They have higher levels of markers of inflammation associated with thrombotic events. These mechanisms may contribute to worse outcomes of saphenous vein graft PCIs of anemic patients.

Our study has several limitations. It is a retrospective single-center study with a relatively small sample size. We did not collect information on the cause of anemia or the presence of conditions predisposing to anemia. We did not record discharge hemoglobin or changes in hemoglobin levels during hospitalization, which were reported to be associated with mortality. Admission hemoglobin levels may fluctuate according to hydration status. We could not analyzed the influence of dual antiplatelet therapy duration after SVG PCI.

In conclusion, baseline hemoglobin levels may predict all-cause mortality in patients undergoing saphenous vein graft interventions. The value of correcting anemia before the procedure should be adressed in prospectively designed trials.

Informed Consent: Due to the retrospective design of the study, informed consent was not taken.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors have no conflicts of interest to declare.

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Original Research

Role of Serum HMGB1 in Prostate Cancer

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ABSTRACT

Purpose: In our study the diagnostic role of HMGB1 levels measured in serum were investigated in prostatitis and prostate carcinoma diagnosis and in the differential diagnosis of these two diseases.

Material Method: Patients followed up for histopathologically verified diagnosis of prostate carcinoma and prostatitis in 2014-2017 at the Urology Clinic were included. HMGB1 measurement in serum was performed with the ELISA method.

Results: A total of 78 subjects were included in the study, consisting of 30 (38.5%) prostatitis patients, 25 (32%) prostate carcinoma patients and 23 (29.5%) healthy subjects. HMGB1 was detected as 11.9±2.6 (Range 6.7-18.4) ng/ml in the prostatitis group, 15.1±4.5 (Range 8.4-24.8) ng/ml in the prostate carcinoma patients and as 9.2±3.1 (Range 4.7-18.7) ng/ml in the control group. The difference between the groups were investigated using the Friedman test as HMGB1 did not show normal distribution. Significant difference was detected between the three groups ($p<0.001$). When the groups were compared in pair, significant difference was detected between the prostatitis group and the control group ($p=0.001$). Significant difference was again detected between the prostate carcinoma group and the control group ($p<0.001$). Significant difference was detected between the prostatitis group and the prostate carcinoma group ($p=0.006$). Measurement of serum total prostate specific antigen (tPSA) levels were conducted automatically with the electro chemiluminescent method. A moderate level of ($r=0.276$) but a highly significant ($p=0.009$) positive correlation was found between PSA and HMGB1.

Conclusion: In our study we showed that high PSA and high HMGB1 were highly correlated. HMGB1 measured in serum could be a useful marker in the differentiation of prostatitis and prostate carcinoma, in the early diagnosis of suspected prostate carcinoma and that HMGB1 value was significantly high in prostate carcinoma patients.

Keywords: Diagnosis, HMGB1, prostate carcinoma, prostatitis, PSA

INTRODUCTION

Prostate carcinoma is the most frequently seen cancer in men in the USA (1). Early stage prostate carcinoma can be cured with radical surgery or definitive radiotherapy. Despite these treatments local or remote relapse can occur in the patients. 10-20% of prostate carcinoma patients are presented with metastatic disease (2). Therefore, early diagnosis of these patients is important.

Prostate specific antigen (PSA) has an important role in prostate carcinoma diagnosis. Abnormal digital rectal examination findings and high serum PSA levels are the most important indicators that cause prostate biopsy indication (3). Increased levels of serum PSA are associated with carcinoma, bacterial prostatitis, prostatic inflammation, benign prostate hyperplasia (BPH) and urinary system infection.

Prostatitis is observed at a rate of 8.2% in men. Acute bacterial prostatitis (ABP) is a pyogenic infection of the urinary system and is seen at a rate of 5% among overall prostatitis patients (4). ABP is most frequently caused by *Escherichia coli; Entero-*

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coccus, Proteus, Pseudomonas, Klebsiella and *Serratia* organisms also cause it less frequently. Prostatitis can cause oedema in the prostate and result in urinary retention and may cause serious complications (5). In early stage treatment, parenteral antibiotics (such as ceftriaxone, aminoglycosides, fluoroquinolone) are given and if the patient cannot perform intravenous hydration and urination then drainage is performed with catheter. PSA values are usually high in case of ABP. Sometimes unexpected high values are detected. This may lead to unnecessary prostate biopsies.

High mobility group box (HMGB) are non-histone nuclear proteins with many functions in the cell. While the expression of HMGB3 and HMGB2 is limited to certain periods of life and to certain cells, the expression of HMGB1 is very prevalent and continues in adulthood (6). HMGB1 acts as a chromatin binder factor. It binds to the small groove of the DNA and modifies the interaction with the DNA of certain transcription factors including p53 and steroid hormone receptors. It plays a role in DNA repair, transcription, differentiation, extra-cellular signalization and somatic recombination. Various strategies, such as HMGB1-receptor antagonists, inhibitors of its signalling pathway, antibodies, RNA inhibitors, vagus nerve stimulation etc. have been used to inhibit expression, release or activity of HMGB1 (7). In addition to these nuclear functions, it also functions as an extra-cellular signaling molecule by being passively secreted from necrosis cells and actively secreted from cells playing a role in inflammation (8).

In our study the diagnostic role of HMGB1 levels measured in serum were investigated in prostatitis and prostate carcinoma diagnosis and in the differential diagnosis of these two diseases and the relation between HMGB1 and PSA were examined.

PATIENTS AND METHOD

Patient Selection

Patients diagnosed with prostate cancer and prostatitis between 2014-2017 in the Urology Clinic were included in the study. Written consent was obtained from the patients. Patient files were screened and information such as age, gender and routine laboratory tests were retrospectively obtained. Patients were divided into risk groups according to Gleason score and PSA levels. Low-risk prostate cancer: T1-T2a stage and Gleason score ≤6 and PSA ≤10, moderate risk prostate cancer: T2b stage and / or Gleason score = 7 and 10≤PSA≤ 20 and high-risk prostate cancer: ≥ T2c stage or Gleason score 8-10 or PSA> 20 **(9).**

Sampling

Blood samples remaining from the blood samples of patients for routine checks were collected prospectively directly before the start of first-line systemic chemotherapy. They were centrifuged for 15 min at 1,000g within 1 hr of collection. The resulting sera were aliquoted into microtubes and either immediately frozen at -80 °C. These samples were placed into a refrigerator at 4 °C one night before the measurements. Serum samples were kept at room temperature for 2 hours before operating with the ELISA method. The samples were then mixed using vortex and measurement procedures were applied.

HMGB1 and PSA Measurement

HMGB1 serum levels were measured using Rel Assay Brand commercial kits and by complying with manufacturer's instructions (Rel Assay Diagnostics® Mega Tıp Ltd, Turkey). Analysis operations were performed by using sandwich enzyme immunoassay technique and by repeating twice for each sample. All concentration/absorption graphic curves of the test and calculations regarding the results were performed on the program of the Biotek_ELx808 (Winooski, Vermont, USA) device. The test was determined to have a sensitivity of 0.06 ng / mL and detection range of 1-32 ng / mL. Intra-assay and inter-assay variation coefficients were determined as 5.7% and 6.3% respectively. Serum total prostate specific antigen (tPSA) values were measured automatically with electrochemiluminescent method by using the Hitachi Modular Analytics E 170 device (Roche Diagnostics GmbH, Germany).

Statistical Analysis

Statistical analyses were performed using the SPSS for Windows 15.0 package software. Compliance of the variables to normal distribution was examined using visual (histogram and probability graphs) and analytic methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). In the Kolmogorov-Simirnov test, cases with p value greater than 0.05 were accepted as normal distribution. Differences between the two groups were tested using the student t test as normal distribution was observed. Differences between the prostatitis, prostate carcinoma patients and the control group were examined using the Friedman test. The patient and control group were compared using the Mann-Whitney U test when normal distribution was not observed. The difference between the groups were investigated using the Kruskal-Wallis test when the variable did not show normal distribution in more than two independent groups. Pair comparisons were made using Mann-Whitney U test and evaluated by using Bonferroni correction. The relation between the HMGB1 and PSA measurements were evaluated with the spearman test. Total type-1 error level was used as 5% for statistical significance.

RESULTS

The study was completed with a total of 78 patients. In these patients, 30 (38.5%) patients had prostatitis, 25 (32%) patients had prostate cancer, 23 (29.5%) patients had healthy volunteers.

The mean age of patients with prostatitis was 60.9±11.2 whereas the mean age of patients with prostate carcinoma was 70.2±6.3. The difference between the two groups in terms of age was statistically significant (p=0.001) (Table 1).

PSA was detected as 45.2±102 (4.9-385) in the prostatitis group and as 141.2±222.7 (Range 6-1200) in the prostate carcinoma group. The difference between the two groups in terms of PSA was evaluated by using the Mann-Whitney U test as the PSA variable did not have a normal distribution. Statistically significant difference was observed between the two groups in terms of PSA levels (p=0.02). PSA value was found to be higher in patients diagnosed with prostate cancer with respect to patients diagnosed with prostatitis (Table 2).

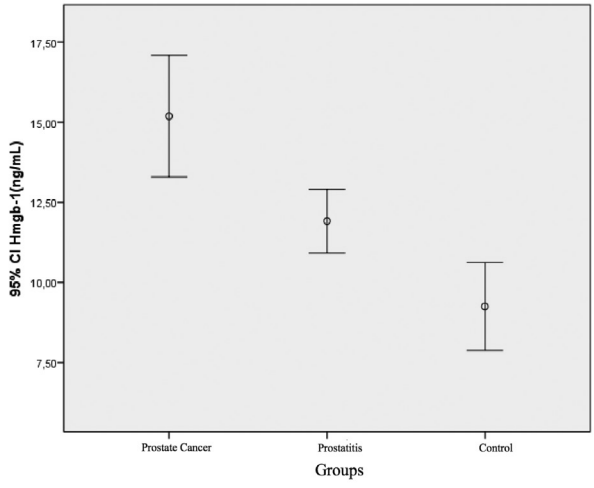
Table 1. Comparison of ages and serum t PSA levels

	Prostate Cancer (n:25)	Prostatitis (n:30)	Control Group (n:23)	P Value
Age(years) Mean±SD (min–max)	70.2±6.3 (55–80)	60.9±11.2 (34–78)	52.3±22.1 (44–76)	0.001
t PSA(ng/ml) Mean±SD (min–max)	141.2±222.7 (6–1200)	45.2±102 (4.9–385)	1.1±3.8 (0.6–3.3)	0.001

Table 2. HMGB 1 levels of groups

	Prostatitis (n:30)	Prostate Cancer (n:25)	Control Group (n:23)
HMGB1 ng/ml Mean±SD (min–max)	11.9±2.6 (6.7–18.4)	15.1±4.5 (8.4–24.85)	9.2±3.1 (4.7–18.7)

Figure 1. Differences between groups



HMGB1 was observed as 11.9±2.6 (Range 6.7-18.4) ng/ml in the prostatitis group, 15.1±4.5 (Range 8.4-24.8) ng/ml in the prostate cancer patients and as 9.2±3.1 (Range 4.7-18.7) ng/ml in the control group. The difference between the groups were investigated by using the Friedman test as HMGB1 did not show normal distribution. Significant difference was found between the groups (p<0.001) (Figure 1). When the groups were compared in pair, significant difference was identified between the prostatitis group and the control group (p=0.001). Significant difference was again observed between the prostate cancer group and the control group (p<0.001). Significant difference was observed between the prostatitis group and the prostate carcinoma group (p=0.006).

The correlation between PSA and HMGB1 was investigated by using the spearman test as the variables did not have normal distribution. A moderate level of (r=0.276) but a highly significant (p=0.009) positive correlation was found between PSA and HMGB1.

When the patients with prostate carcinoma were evaluated according to Gleason score and PSA, 3 (12%) had low risk, 8 (32%) had moderate risk and 14 (56%) had high risk. The relation between the HMGB1 and prostate cancer risk groups was investigated by using the Kruskal-Wallis test. Significant difference was not observed between the three groups (p<0.352).

DISCUSSION

PSA is the most frequently used biochemical marker in prostate carcinoma diagnosis. PSA levels are used in both treatment evaluation and in follow-up (10). Serum PSA levels can be detected at highly varying levels in bacterial prostatitis patients. Many studies have shown that PSA levels returned to normal after treatment of prostatitis (11). In our study we have shown that HMGB1 levels measured in serum can play a role in the differential diagnosis of prostate carcinoma and prostatitis.

High mobility group box (HMGB) are non-histone nuclear proteins with many different functions in the cell. HMGB proteins were first purified from the nucleus in the 1970s. They were named as such due to their fast movement in the sodium dodecyl sulfate polyacrylamide gel electrophoresis when they were first discovered (6). HMGB1, HMGB2 and HMGB3 are members of the HMGB protein family. HMGB2 and HMGB3 have limited expression while HMGB1 has prevalent expression and its expression can be regulated with environmental factors. HMGB2 is expressed at high ratios during embryogenesis and in contrast with HMGB1, its expression in adulthood is limited to the lymphoid organs and the testis. The common and differential functions of HMGB1 and HMGB2 proteins with reference to pathological processes, with a special focus on cancer (12). HMGB1 binds to the small groove of the DNA and modifies the interaction with the DNA of certain transcription factors including p53 and steroid hormone receptors. It plays a role in DNA repair, transcription, cellular differentiation, extra-cellular signalization and somatic recombination. HMGB-1 is considered as an essential facilitator in diseases such as sepsis, collagen disease, atherosclerosis, cancers, arthritis, acute lung injury, epilepsy, myocardial infarction, and local and systemic inflammation. Modulation of HMGB1 levels in the human body provides a way in the management of these diseases. Various strategies, such as HMGB1-receptor antagonists, inhibitors of its signalling pathway, antibodies, RNA inhibitors, vagus nerve stimulation etc. have been used to inhibit expression, release or activity of HMGB1 (7). In addition to these nuclear functions, it also functions as a signal molecule and acts as an extra-cellular signal molecule in inflammation, cellular differentiation, cell migration and tumor metastasis (13). HMGB1 has high bonding affinity to certain receptors by being passively secreted from necrotic cells and actively secreted from

inflammatory cells. These include RAGE (receptor for advanced glycation end products) and Toll-like receptors (TLR)-2, TLR-4, TLR-9 (14).

The relation between prostate carcinoma and HMGB1 expression was investigated in many studies. Li et al. showed both HMGB1 mRNA and protein expression with their polymerase chain reaction and western blotting method in carcinoma cell cultures in prostate (15). Using immunohistochemical (IHC) method they showed HMGB1 expression in tumor tissue cell samples of 168 prostate carcinoma patients obtained with prostatectomy. In their studies they detected that HMGB1 protein expression was high in tissue samples and that this high level was correlated with both the Gleason score and the pre-operative PSA concentration.

In their studies Gnanasekar et al. investigated the role of HMGB1 in the development of prostate carcinoma (16). In this study they showed that HMGB1 had an important role in the expression and upregulation of androgynous receptors in prostate carcinoma patients. In their studies Zhang et al. reported that HMGB1 was highly expressed in metastatic prostate cancer samples and able to enhance the aggressiveness of PC3 cells. HMGB1 promotes the EMT process and upregulates the expression levels of MMP-1, -3 and -10 by activating the RAGE/NF-κB signaling pathway in PC3 cells, thereby facilitating cancer metastasis. (17).

These studies strongly indicate that HMGB1 could have an important role in the progress of prostate carcinoma. Zhao et al. on the other hand investigated HMGB1 and RAGE expression using IHC in tissue samples taken after prostatectomy from 85 prostate cancer patients (18). In this study they could not show the relation of both HMGB1 and RAGE expression with Gleason score. However, they asserted that HMGB1 expression was a poor prognostic marker and could be used as a new prognostic marker for prostate carcinoma. In our study we could not show the relation between HMGB1 levels measured in serum and risk groups that mostly guide for prostate carcinoma treatment.

There are few studies investigating HMGB1 expression in prostatitis patients. Xue et al. compared HMGB1 expression using IHC method in tissue samples of BPH patients with prostatitis and BPH patients without prostatitis (19). In this study they detected HMGB1 extraction to be at a higher ratio in BPH patient.

The limitations of our study is that the number of samples was small and the study was conducted retrospectively.

In our study we showed that high PSA and high HMGB1 levels measured in serum were highly correlated and that HMGB1 value was apparently higher in prostate carcinoma patients. We consider that HMGB1 measured in serum could be a useful marker in the differentiation of prostatitis and prostate carcinoma, in the early diagnosis of suspected prostate carcinoma cases.

Abbreviations
HMGB1**:** High mobility group box-1
PSA: Prostate specific antigene
ABP: Acute bacterial prostatitis
DNA: Deoxyribonucleic acid
RAGE: Receptor for advanced glycation end products
TLR-2: Toll-like receptors-2
TLR-4: Toll-like receptors-4
TLR-9: Toll-like receptors-9
mRNA: mesenger ribonucleicacid
IHC: Immunohistochemical
BPH: Bening prostate hyperplasia

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Local Ethical Committee (04/29.03.2018).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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Original Research

Investigation of Various Virulence Factors and SCCmec Types in the Healthcare-associated and Community-associated Methicillin Resistance Staphylococcus aureus Strains

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ABSTRACT

Objective: The objective of this study was to investigate some virulence genes and SCCmec types of *methicillin-resistant Staphylococcus aureus* (MRSA) isolates and to determine their relationship with virulence factors.

Methods: A total of 100 MRSA strains, 64 from healthcare-associated and 36 from community-associated infections, were included in the study. The presence of mecA gene was investigated by PCR. SCCmec types and efb, clfB, agrA gene were detected by multiplex PCR and their relationship with virulence factors has been analyzed.

Results: All of the isolates contain the mecA gene. At the same time, in 66 strains (66%) agrA gene, in 58 strains (58%) clfB gene, and in 47 strains (47%) efb gene were positive. In terms of SCCmec types, the distribution of these types among the 64 HA-SA strains was 53% similar-to-type-III, 16% type IV, 2% type I and 30% unclassified. The distribution of the types among the 36 CA-SA strains was 19% similar-to-type-III, 25% type IV, 8% type I and 47% unclassified, respectively. When SCCmec types were evaluated according to clinical sample type, similar-to-type-III isolates were found to be dominant in wound samples. Efb (78%), clfB (85%), agrA (88%) were the dominant genes in similar-to-type-III strains, whereas clfB (74%), agrA (100%) were the main genes detected in the type IV strains.

Conclusions: It is of clinical and epidemiological importance to know the origin of MRSA strains because this affects the empirical treatment choice.

Keywords: *Methicillin-resistant Staphylococcus aureus,* PCR typing, SCCmec, virulence factors

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium that causes epidemics and endemics worldwide and leads to infections with high morbidity and mortality (1).

S. aureus is the most commonly isolated bacterium in both community-associated (CA-SA) and healthcare-related (healthcare-associated-HA-SA) infections. *S. aureus* can cause serious infections, such as life-threatening pneumonia and toxic shock syndrome from skin and soft tissue infections (2). The first MRSA strains associated with healthcare delivery were identified in 1960 and CA-MRSA was first described in 1980. These strains are primarily associated with skin and soft tissue infections, but now also cause health-related infections (1,3).

In addition to the increase in the prevalence of hospital infections caused by CA-MRSA, there is the issue that it cannot be easily differentiated from HA-MRSA, based on clinical and epidemiological criteria. For this reason, the use of genetic indicators in their classification has gained importance (4). MRSA strains

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isolated from community-associated infections, have been observed to be different from HA-MRSA both genotypically and phenotypically (3).

Polymerase chain reaction (PCR) is frequently used in the detection of methicillin resistance, genotyping of MRSA strains and determining virulence factors (5).

The mecA gene encoding the methicillin resistance is located on a mobile genetic element called mec (SCCmec) of the staphylococcal cassette chromosome. SCCmec consists of mec gene complex (mecA and regulating genes) and ccr complex. To date, 13 (I-XIII) main types have been defined in different SCCmec types. Of these, SCCmec type I, II and III were mostly detected in HA-MRSA; Type IV, V, VI, and VII were associated with CA-MRSA strains (3,6).

In *S.aureus* infections, virulence factors that are found on the cell surface and secreted out of the cell also play an important role. The virulence of *S.aureus* strains does not depend on any of these biological factors alone but is caused by several effects (7). Different virulence markers are observed at different stages of staphylococcal infections. Agr (accessory gene regulator) is a core sensor system that plays a critical role in the systemic infection process. Agr, a quorum-sensing system, plays a role in the regulation of transcription of genes encoding some surface proteins and enzymes released outside the cell (8).

Whether all MRSA strains have an equal potential for disease or whether invasive and chronic diseases are associated with virulent genotypes is still unknown. The identification of virulence genes may explain this issue (1).

The aim of this study was to determine the SCCmec types of MRSA strains isolated from outpatients and outpatients by multiplex PCR and to determine the distribution of some important virulence genes among these types.

MATERIALS AND METHODS

Patient groups and bacterial strains

One hundred MRSA strains from various polyclinics/services from the Microbiology Laboratory in 2014-2016 were included in our study. The strains were identified by the Phoenix ™ 100 system (Becton Dickinson, USA) and confirmed by the coagulase test. If more than one MRSA was isolated from one patient, only one was included in the study. The strains were stored at -80 °C in a Brain-Heart Infusion medium with 10% glycerin.

Infection origin types were defined according to CDC criteria (9). Thirty-six of all strains were isolated from patients admitted to various polyclinics of the hospital and these strains were accepted as 'community-acquired'. The other 64 strains were isolated from the different infection sites of patients in the hospital and in the intensive care units, and these strains were accepted as 'healthcare-associated'. The clinic, sample type and date information of patients were recorded. When evaluated in terms of sample type, 58 of the strains were blood, 20 were wound, 9 were nasal, 7 were urine and 6 were sputum samples.

Determination of methicillin resistance

Methicillin resistance in strains was determined by detecting the minimum inhibitory concentration (MIC) of cefoxitin by the Phoenix ™ 100 (Becton Dickinson, USA) automated system. Data were confirmed by the cefoxitin disc diffusion (DD) test according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (10) standards. *S. aureus* ATCC 25923 was used as a control group.

Antibiotic susceptibility testing

The in-vitro susceptibilities of the strains to gentamicin, levofloxacin, tetracycline, erythromycin, clindamycin, trimethoprim-sulfamethoxazole, rifampicin, vancomycin, teicoplanin, daptomycin, quinupristin-dalfopristin, and linezolid were also determined by the Phoenix ™ 100 (Becton Dickinson, USA) automated system.

DNA isolation

Prior to PCR experiments, genomic DNA was isolated as in the Merlino et al. (11) study.

Investigation of virulence genes and *mecA* gene

The presence of the *mecA* gene in resistant strains was investigated. In addition, the presence of the *agrA* regulatory gene, responsible for the regulation of various toxins, enzymes and surface proteins and *efb* (extracellular fibrinogen binding protein) *clfB* (cloning factor B) virulence genes were investigated using the multiplex PCR method. The thermal cycling conditions were applied using the primers indicated in Table 1 for 1 minute at 94°C, 1 minute at 42°C and 1.5 minutes at 72°C, following 5 minutes initial denaturation at 94°C. Finally, multiplex PCR was completed with final elongation for 10 minutes at 72°C.

Molecular typing with multiplex PCR

In order to determine the SCC*mec* types of strains, primers given in the study performed by Milheiricho et al. (12) were used. Thermal cycling conditions were also applied as given in the same study. *S. aureus* HPV107 (SCCmec type IA), BK2464 (SCCmec type II), HUSA304 (SCCmec type III), HSJ216 (SCCmec type IIIA) and GRE14 (SCCmec type IV) were used as a positive control.

Analysis of Results

Statistical significance was analyzed by Fisher's exact chi-square test using the GraphPadPrism (California, USA) program. If *p*-value ≤0.05, the results were considered significant.

RESULTS

45 (45%) of the strains included in the study were isolated from females and 55 (55%) were from males. The mean age was 62.25 (24-99) years. *S. aureus* strains were isolated from blood (58; 58%), wound (20; 20%), nasal (9; 9%), urine (7; 7%) and sputum (6; 6%) samples. Hospital-associated strains were most frequently isolated from blood samples (50/64, 78.12%) and community-acquired strains were isolated from wound samples (20/36, 55.55%). 29 (29%) of the strains were from intensive care units (anesthesia, neurology, internal medicine, brain surgery, cardiology), 24 (24%) from internal medicine clinic/polyclinic, and 22 (22%) other services / polyclinics (neurology), dermatology, chest, infectious diseases, 17 (17%) from surgical services / poly-

Table 1. Virulence genes and primers used in searching the mecA gene

Target gene	Length (bp)	Primers	Reference	
clfB	596	F	5'–TGCAAGATCAAACTGTTCCT–3'	11
		R	5'–TCGGTCTGTAAATAAAGGTA–3'	
efb	434	F	5'–TAACAATAGCGGCAATAGGT–3'	This study
		R	5'–CAATTCGCTCTTGTAAGACCA–3'	
agrA	193	F	5'–TCACAGACTCATTGCCCATT–3'	12
		R	5'–CACCGATGCATAGCAGTGTT–3'	
mecA	300	F	5'–TGCTATCCACCCTCAAACAGG–3'	13
		R	5'–AACGTTGTAACCACCCCAAGA–3'	

Table 2. Distribution rate of genes in terms of being hospital and community associated

	HA(%)	CA(%)
efb	42	48
clfB	56	59
agrA	56	70

Table 3. Multiplex Amplification Patterns of 36 CA–MRSA Origin Multiplex Pattern and Interpretation

Number of Strains	Multiplex Pattern
22	Only mecA band
5	Type I pattern without J3 region (342 bp)
4	Type III pattern without a J1 region (243 bp) and mec complex (209 bp)
2	J1 region (495 bp), ccr complex (449 bp), J3 region (414 bp) and mecA band (162 bp) together
1	Type III pattern with a J1 region (495 bp)
1	J1 regions (495 bp), ccr complex (449 bp) and mecA band (162 bp) together
1	ccr complex (449 bp), a ccr complex (311 bp) and mecA band (162 bp) together

clinics (general surgery, neurosurgery, urology, orthopedics, otolaryngology, cardiovascular surgery) and 8 (8%) from emergency services obtained from clinical samples sent from the observation unit.

Prevalence of Virulence Genes and mecA Resistance Gene

According to the multiplex PCR results, it was observed that all of the strains (100) included in the study carried the mecA resistance gene. The distribution rates of virulence genes investigated in HA and CA strains are given in Table 2.

Statistically, there was no significant difference in terms of the distribution rates of these virulence genes between the two groups ($p > 0.05$).

Distribution of SCCmec Types

In 41 (41%) of all strains, in addition to bands of 414, 243, 209 and 162 bp, which form the type III pattern, a band of the size of 342 bp, which normally corresponds to the dcs region of type I, II, IV and V was observed. Gülmez et al. (13) who benefited from the previous study of the same author group (14) identified 342 band size bands corresponding to the dcs region and named the similar- to- type III pattern. In addition, 19 (19%) strains were classified as type IV and 4 (4%) as type I, 4 of whom belonged to type IVe subtype.

In this study, a variety of patterns have been observed in 14 of the 36 strains with non-detectable SCCmec types, such as the absence of some loci that cause the pattern of a particular type to be missing or the combination of specific loci of different types, a band with only 162 bp internal positive control amplicon (mecA) was observed. The patterns shown by non-groupable strains are shown in Table 3.

The distribution of SCCmec types in 64 hospital-associated strains was determined as 53% similar-to-type-III, 16% type IV, 2% type I and 30% non-groupable. The distribution of SCCmec types in 36 community associated strains was determined as 19% similar to type III, 25% type IV, 8% type I and 47% non-groupable.

Upon examining the distribution of types in terms of strain type, similar-to-type-III strains were found to be frequent in the wound samples ($p < 0.05$), and there was no statistically significant difference between the groups in the distribution of other types ($p > 0.05$).

Similar-to-type-III strains were found to have efb (78%), clfB (85%) and agrA (88%) genes. For type IV strains, on the other hand, clfB (74%) and agrA (100%) genes was detected. The distribution of these genes was found to be much less frequent in Type I strains and non-grouped strains.

Figure 1. An example of SCCmec typing results of isolates. M: DNA marker, lane 75: similar-to-type-III, lane 76: similar-to-type-III, lane 77: similar-to-type-III, lane 78: only mecA, lane 80: type IV, lane 81: subtype IVe, lane 82: similar-to-type-III, lane 83: only mecA, lane 84: similar-to-type-III, lane 85: type I pattern without J3 region (342 bp), lane 86: similar-to-type-III, lane 87: similar-to-type-III, lane 88: similar-to-type-III, lane 89: similar-to-type-III, lane 90: similar-to-type-III, lane 92: only mecA, lane 93: similar-to-type-III, lane 94: type I pattern without J3 region (342 bp).

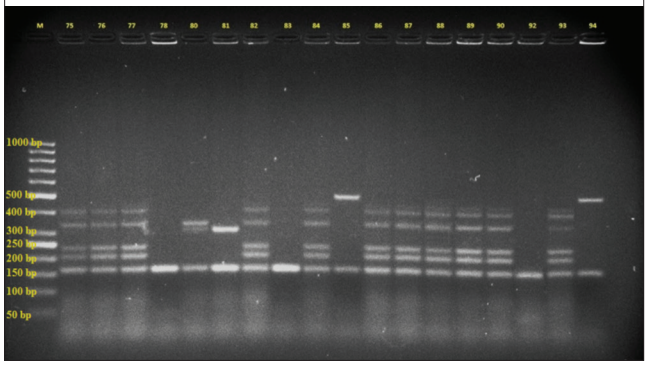
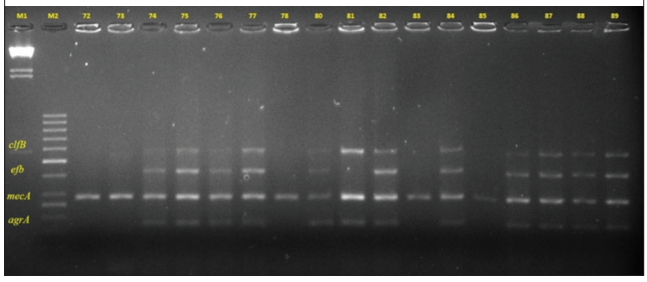


Figure 2. Multiplex PCR for virulence genes (clfB, efb, agrA) and mecA.



Antibiotic susceptibility testing

All *S. aureus* strains were susceptible to vancomycin, teicoplanin, daptomycin, quinupristin-dalfopristin and linezolid. Other antibiotic resistance rates of *S. aureus* strains with HA/CA were; gentamicin 50.0%/33.3%, levofloxacin 64.06%/26.7%, tetracycline46.7%/33.3%, erythromycin 64.06%/53.3%, clindamycin 46.7%/20%, trimethoprim-sulfamethoxazole 34.4%/33.3%, rifampicin 48.4%/20%.

DISCUSSION

Methicillin-resistant *S. aureus* infections continue to be a significant threat to human health in the second decade of the 21st century. Despite significant progress in understanding MRSA infections and virulence mechanisms, there are continuing challenges that need to be addressed. Knowledge of the prevalence of genetic markers and virulence factors contributing to the success of MRSA will be useful for the control and treatment of community and hospital induced *S. aureus* infections.

HA-MRSA is seen to occur in different rates in our country in relation to the regions. Alp et al. (15) reported that HA-MRSA prevalence ranged between 12-75% in a multicentered study they carried out in Turkey, in eight university hospitals, in six different geographic regions, where they had isolated MRSA. The prevalence of CA-MRSA was reported to be 1-3% in our country (16).

In addition to the genotypic differences between HA-MRSA and CA-MRSA, the strains affect different populations and cause different clinical syndromes. CA-MRSA infections tend to occur in healthy children and young adults and are associated with skin and soft tissue infections including necrotizing fasciitis and severe invasive infections such as pneumonia and sepsis. In contrast, HA-MRSA strains are most commonly seen in patients who are under antibiotic treatment, have a weakened immune system and who have been treated using invasive medical devices. HA-MRSA strains commonly cause pneumonia, bacteremia and invasive infections (17). In our study, MRSA strains were first isolated from blood (58%) and secondly from wounds (20%) according to sample types. HA-strains were most frequently isolated 78.12% from blood samples and 55.55% CA -strains were isolated from wound samples.

The risk of bacteremia due to MRSA in inpatients varies according to the services. The highest risk is stated to be in intensive care units (18). In our study, when the distribution of MRSA strains according to services was examined, it was seen that 29% of them were isolated from intensive care. This may be due to a more invasive procedure, more severe underlying disease and / or the presence of immunosuppression.

The monitoring of the SCC mec has been carried out around the world for many years. In MRSA strains, it has been confirmed that methicillin resistance gained through the ccr and mec gene complex (mecA and new homologs; mecB, mecC, mecD), influences the concentration of beta-lactam antibiotic resistance and antimicrobial minimal inhibitor, which has been found to lead to multiple drug resistance (19). HA-MRSA strains are resistant to multiple drugs due to drug-resistant genes integrated into SCCmec (20,21). CA-MRSA strains are generally susceptible to non-beta-lactam antibiotics because they do not carry the resistance gene other than mecA (3).

A thorough understanding of the prevalence and occurrence of SCCmec may help further to identify, control, prevent and treat staphylococcal-mediated human diseases (19). In the studies in order to type SCCmec in MRSA strains in Turkey, more than 80% of HA-MRSA strains were reported to be SCCmec type III, rarely SCCmec type IIIb, CA-MRSA strains were reported to be SCCmec type IV and V more frequently, SCCmec type I and II have been reported less frequently (15). Karahan et al. (22), reported SCCmec type I or II or III and subtypes in 99% of HA-MRSA strains, SCCmec type IV in 1%; In 60% of CA-MRSA strains, SCCmec type I or II or III and 40% SCCmec type IV or V; Tekeli et al. (23) reported 84% SCCmec type III in MRSA strains isolated from blood cultures of hospitalized patients; Kılıç et al. (16), reported SCCmec type I or II in 3.6% of MRSA strains, SCC mec type III in 82.1%, SCCmec type IV in 5.1% and SCCmec type V in 5.1% of MRSA strains; Gülmez et al. (13) SCCmec type IVa in CA-MRSA strains, similar to SCCmec type III in HA-MRSA strains; Akoğlu et al. (5), 61.8% SCCmec typeIII in HA-MRSA strains, 34.5% SCCmec type IIIb and 2.7% SCC-

mec typeIV; Baran et al. (24) found SCCmec type III in 24 (85.7%) of the HA-MRSA strains and 100% SCCmec type IV in all CA-MRSA strains, 1 (3.6%), SCCmec type IV, and 3 (10.7%) HA-MRSA strain could not be typed by the method used. In 2013, Oksuz et al. (25) SCCmec type III in MRSA clones; Yilmaz et al. (26) in the HA-MRSA strains, 90% SCCmec type III, 2.2% (1 isolate) SCCmec type IV and 40% SCCmec type IV were detected in CA-MRSA strains and they could not make the typing of the remaining strains. Similar to other studies conducted in our country, 53% SCCmec type III strains were found most frequently in HA-MRSA, while 25% type IV strains and 47% non-groupable strains were found to be the most common in CA-MRSA. Similar-to-type-III strains were found to be predominant in wound samples.

It was thought that community-associated MRSA was initially from nosocomial strains and was spreading from hospitals to the community. However, paradoxically, the sensitivity of CA-MRSA to non-beta-lactam antimicrobial agents and their association with clinical syndromes typical for Methicillin-sensitive *Staphylococcus aureus* (MSSA) are strong evidence that CA-MRSA is different from the strains seen in health care units. After the introduction of genotypic differences that differentiate CA-MRSA from HA-MRSA, the idea that CA-MRSA develops from MSSA, which is endemic in the community, has started to be accepted generally. The beta-lactams, once effective in community-associated *S. aureus* strains, have transformed into unreliable therapeutic agents. CA-MRSA is often more sensitive than HA-MRSA to non-beta-lactam antibiotics such as clindamycin, TPM / SMX, and doxycycline (2). Many MRSA clones have gained resistance to antibiotics such as erythromycin, clindamycin, ciprofloxacin, tetracycline. Multi drug resistance exists (27). Most of the CA-MRSA strains were not resistant to additional antibiotics except for the limited outbreaks of multidrug-resistant CA-MRSA (28). In studies conducted in our country, Akoğlu et al. (5) reported a high (> 90%) resistance to gentamicin, ciprofloxacin, and rifampicin, sensitivity to TMP-SMX 90%, clindamycin 53% and erythromycin 32%. Öksüz et al. (25) found penicillin 100%, tetracycline 100%, rifampicin 100%, kanamycin, tobramycin, 93%, levofloxacin 93%, erythromycin 75%, lincomycin 49%, phosphomycin 58% and fusidic acid 4% multidrug resistance. Baran et al. (24) found susceptibility to vancomycin, linezolid and TMP-SMX in all strains, whereas in HA-MRSA / CA-MRSA strains respectively; rifampicin 89.3% / 0%, ciprofloxacin 89.3% / 50%, gentamycin 89.3% / 0%, erythromycin 50% / 50% and clindamycin 28.6 / 0% rates of resistance were found. Tekeli et al. (29); found susceptibility to vancomycin in all strains, 97.7% in tetracycline, 97% in ciprofloxacin, 100% in rifampicin and 94.7% in gentamicin.

In our study, all *S. aureus* strains were found to be sensitive to glycopeptides. Other antibiotic resistance rates of *S. aureus* strains in hospital/community associated strains were 50.0% / 33.3% gentamicin, 64.06%/26.7% levofloxacin, 46.7% / 33.3% tetracycline, 64.06% / 53.3% erythromycin, 46.7% / 20% clindamycin 34.4% / 33.3%, 48.4% / 20%. TMP-SMX rifampicin. It was observed that our resistance rates were lower than other centers.

In recent years, CA-MRSA strains may also have played a role as a nosocomial infectious agent (30). Type IV SCCmec is primarily associated with MRSA infections in patients without risk factors for HA-MRSA. However, according to recent data on patients who do not have risk factors for HA-MRSA, most of the hospitalized patients in the US now have SCCmec IV (31). Gonzalez et al. (32) found SCCmec type IV in 60% of the HA-MRSA strains isolated from blood.

The virulence factors in *S. aureus* infection have gained importance. There are studies on the activity of agr, clf, efb virulence genes (28,33,34). The host factors that affect the severity of the disease remain an unexplained subject. In understanding virulence factors regulators more research is needed to determine how virulence factors are transmitted between MRSA strains (1).

In our study, it was observed that in type III strains efb78%, *clfB* 85% and *agrA* 88% genes, whereas in type IV strains clfB and 74% and agrA 100% genes were found to be frequent respectively. The distribution of these genes was found to be much less frequent in Type I strains and non-grouped strains.

CONCLUSION

The selective pressure of antimicrobial agents, together with the acquisition of genetic markers, allows MRSA to adapt to different environmental conditions, leading to a global spread of MRSA. Genetic backgrounds that can allow for the development of the MRSA clones can be determined through population studies and post-genomic investigations. The response of regulatory systems to external signals is a significant element in the epimediologic success of a particular clone. Thus, as has been suggested by many recent studies, these events could be major areas for the development of future therapeutic interventions. Despite the fact that the relationship between endurance, virulence and antibiotic resistance is still to be fully understood, an understanding of how molecular markers can allow the spread of pathological processes will help in the development of prevention and treatment strategies aimed at overcoming the growing challenges associated with MRSA.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Non-Interventional Ethics Committee (1700049757/31.07.2017).

Informed Consent: Due to the in-vitro design of the study, informed consent was not taken.

Peer-review: Externally peer-reviewed.

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Original Research

The Relationship between Mitral Chordae Rupture and Inflammation Level and Oxidative Stress

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ABSTRACT

Objective: There are many factors associated with chorda tendinea rupture. The present study aims to investigate the role of Interleukin-6 (IL-6), total antioxidant activity (TAA), total oxidant activity (TOA) and tumor necrosis factor alpha (TNF- α) levels on the development of the mitral valve chordae tendinea rupture.

Methods: Our study consisted of 30 patients with mitral chordae rupture, 30 patients with severe rheumatic mitral regurgitation, and 20 healthy participants who were admitted to our polyclinic unit. The participants do not have a previously known comorbid disease. After transthoracic echocardiography, their diagnoses were confirmed by the transesophageal echocardiography. Plasma IL-6, TAA-TOA and TNF-α level of all patients were measured and their oxidative stress index (OSI) was calculated.

Results: Compared to the rheumatic severe mitral insufficiency patients and control group, TOA-OSI levels in the chordae rupture group were significantly higher (p=0.038 and p=0.019, respectively). The TNF-alpha levels in the rheumatic severe mitral insufficiency group were determined as statistically and significantly higher than the chordae rupture group (p=0.028).

Conclusion: We have put forth that there is a significant relationship between the chordae rupture levels and oxidative stress levels (TOA-OSI) for the first time in our study. According to the results of our study, high oxidative stress might be accepted as a risk factor for chordae rupture. In addition, it has been observed that the TNF-α level in the rheumatic severe mitral insufficiency group have been higher than those in the chordae rupture group. It seems, these data support the role of inflammation in the rheumatic severe mitral insufficiency development.

Keywords: Chordae rupture, IL-6, mitral valve, oxidative stress, TNF-α

INTRODUCTION

Chordae tendinea rupture (CTR) is increasingly reported as an important cause of mitral regurgitation (1). Mitral valve prolapse (MVP), rheumatic valve disease, calcific-degenerative valve disease and infective endocarditis (IE) have been reported as leading causes of chorda tendinea rupture (2). The underlying causes of chordae tendinea rupture and their frequencies vary.

Today more than a hundred diseases are associated with free oxygen radicals. Total oxidant activity (TOA) is a parameter which represents the total amount of all oxidants in a sample. Total antioxidant activity (TAA) is a parameter indicating the total amount of all antioxidants in a similar way. The oxidative stress index value is calculated by dividing these values to each other (3).

IL-6 is pleiotropic cytokine produced by T cells, lymphocytes, fibroblasts, adipocytes, macrophages and endothelial cells (4). IL-6 has both endocrine and paracrine effects, and stimulates platelet aggregation along with tissue factor, CRP and the expression of fibrinogen (5). Several studies showed that the inflammatory component in atherosclerosis may contribute to increased risk for cardiovascular disease (CVD). IL-6 and TNF-α assumed as key pro-inflammatory and immune-stimulatory cytokines for CVD and the metabolic syndrome. TNF-α has also been implicated in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis, myocardial infarction, heart failure, myocarditis and cardiac allograft rejection.(6).

TAA, TOA, IL6 and TNF-α are thought to increase the tissue damage. In this study, we planned to seek the relationship between IL-6, TNF-α, TAA, TOA and mitral valve chordae rupture.

METHODS

30 patients having mitral chordae rupture (group 1), 30 patients with severe rheumatic mitral regurgitation (group 2) and 20 healthy participants (group 3) admitted to the Gaziantep Uni-

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Table 1. Demographic assessment of patients

	Chordae rupture (n=30)	Rheumatic MR (n=30)	Control (n=20)	p
Age (years)	51.1±19.8	45.2±15.6	43.8±12.4	0.362
BMI (kg/m^2)	26.5±3.1	25.5±3.2	24.5±3.9	0.726
Systolic blood pressure (mm Hg)	124.0±10.2	118.6±9.8	120.6±8.8	0.782
Diastolic blood pressure (mm Hg)	78.0±7.1	70.9±9.7	74.9±7.7	0.564

Abbreviations: BMI: Body mass index, MR: Mitral regurgitation

versity Medicine Faculty Cardiology polyclinic for various reasons between 1 June 2014 and 31 January 2015 included to this study. The participants do not have a previously known comorbid disease. The study was approved by the Gaziantep University Medicine Faculty Ethics Committee. All patients and participants provided written informed consent.

Exclusion criteria were previous acute coronary syndrome, coronary artery disease, dilated cardiomyopathy, smoking, autoimmune diseases, pregnancy, diabetes mellitus, rheumatologic diseases, ejection fraction less than 50%, chronic renal or liver disease, active malignancy, vitamin or antioxidant replacement therapy, and known chronic diseases.

Age, sex, body mass index, drug therapy, hematological and biochemical results were recorded. Blood samples were obtained to measure IL-6, TNF-, TAA, TOA and OSI. The OSI was defined as the ratio of the TOA level to TAA level.

All patients underwent echocardiography to assess left ventricular systolic, diastolic functions and dimensions. Ejection fraction by Simpson method, volumes, tissue Doppler parameters (S, e, a), mitral valve PW parameters (E, A) were recorded, grading of mitral insufficiency was assessed by PISA method, vena conracta size and jet area/left atrial area (7).

Laboratory methods

All blood samples were collected to measure IL-6, TNF-α, TAA, TOA and OSI during 1 June 2014-February 2015. Serum was separated and stored at – 70 C within 30 min of collection. The reagents and serum samples were allowed to come to room temperature. TAS - TOS reagents were charged to spectrophotometry (Tokyo Boeki Medical System, Japan). After controlling the calibration device to the examples given device. After operation of the sample results were printed. Patients and controls TNF-alpha and IL-6 serum levels were measured using a human ELISA kit (Shanghai YeHua Biological Technology). This double antibody kit to measure serum levels of TNF- alpha is to use the sandwich ELISA technique. Serum TNF-alpha and IL-6 levels in patients and controls were analyzed by standard graphics.

Statistical analysis

All statistical tests were carried out using SPSS 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Continuous data are expressed as mean ± standard deviation and categorical data are expressed as percentages. Kolmogorov-Smirnov test was used to test the distribution type. Normal distributed variables were compared with One Way ANOVA test and appropriate post-hoc analysis. Not normal distributed variables were compared with Kruskal–Wallis test. Mann Whitey U test was used for the post-hoc analysis. The x^2-test was used to assess differences in categorical variables between groups. The relationship between quantitative variables was tested with the Spearman rank correlation coefficient. The results are expressed as relative risk and 95% confidence interval (CI). A p-value less than 0.05 considered statistically significant.

RESULTS

First, these subjects were divided into three groups: chordae rupture group (n = 30), rheumatic mitral regurtation group (n = 30) and control group(n=20). The demographic characteristics of the patients are shown in Table 1. Statistically, there was no significant difference between the groups in age, gender and systolic and diastolic blood pressure.

Statistically, there was no significant difference between all groups (group 1, 2 and 3) in laboratory parameters (p>0,05). These results of the patients are shown in Table 2.

Compared with the rheumatic severe mitral insufficiency patients, the TOA and OSI levels in the chordae rupture group(group 1) have been found to be significantly high in terms of statistics (p=0.038, p=0.026 respectively). Compared with the control group (group 3), the TOA and OSI levels in the chordae rupture group have been found to be significantly high in terms of statistics (respectively p=0.019 and p=0.023). Group 1 and group 3 patients serum IL-6, TNF-α, TAA, TOA and OSI values shown in table 3. Statiscally there was no significant difference between both groups (group 1 and 3) regarding TAA, IL-6 and TNF-alfa (p>0,05).

The TNF-alpha levels in the (group 2) have been determined as statistically and significantly higher than the levels in the chordae rupture group (p=0.028). Group 2 and group 3 patients serum IL-6, TNF- α, TAA, TOA and OSI values shown in table 3. Statiscally there was no significant difference between both groups (group 2 and 3) regarding TAA, TOA, OSI, IL-6 and TNF-alfa (respectively p=0,169, p=0,566, p=0,714, p=0,488 and p=0,303).

All groups' echocardiographic values were shown in Table 4. Statically, there was no significant difference between groups regarding ejection fraction. But **c**ompared with the control group, the left ventricle(LV) end-diastolic volume, LV end-systolic volume, LV end-diastolic diameter, left atrium(LA) diastolic volume,

Table 2. Comparison of laboratory parameters

	Chordae rupture (n=30)	Rheumatic MR (n=30)	Control (n=20)	p
Hemoglobin (g/dL)	13.2±1.7	13.6±1.9	13.9±2.1	0.742
Hematocrit (%)	38.2±5.4	40.0±6.2	42.1±4.2	0.550
Platelet (/μ)	242.2±94.4	267.9±84.7	288.7±64.7	0.480
MCV (fL)	86.6±7.9	83.9±5.9	84.9±5.5	0.562
WBC (/μ)	8.6±3.0	7.8±2.4	8.8±2.1	0.724
Triglyceride (mg/dL)	159.4±72.1	156.4±102.2	162.4±62.1	0.664
Creatinine (mg/dL)	0.70±0.3	0.68±0.4	0.72±0.4	0.840
LDL (mg/dL)	104.0±29.2	118.1±35.3	108.1±34.9	0.736
HDL (mg/dL)	43.2±11.7	43.7±8.2	44.7±6.2	0.852

Abbreviations: WBC: White blood cell, MCV: Mean cell volume, LDL: Low density lipoprotein, HDL: High density lipoprotein, MR: Mitral regurgitation

Table 3. Comparison of inflammatory parameters

	Chordae rupture (n=30) (group 1)	Rheumatic MR (n=30) (group 2)	Control (n=20) (group 3)	p
TAA	2.19±0.27	2.21±0.36	2.09±0.18	0.370
TOA	11.73±15.83 *,#	6.95±5.71	5.68±3.10	0.032
OSI	0.50±0.61*,#	0.31±0.24	0.27±0.15	0.029
IL–6	96.87±109.18	101.89±82.69	64.42±23.20	0.428
TNF–α	104.25±107.27	125.89±95.99 #	81.25±32.10	0.049

Abbreviations: TAA: Total antioxidant activity, TOA: Total oxidant activity, OSI: Oxidative stress index, IL–6: Interleukin–6, TNF– ALFA: Tumor necrosis factor alpha, MR: Mitral regurgitation
*p<0.05 for chorda rupture vs control group
#p<0.05 for chorda rupture vs rheumatic MR group

LA systolic volume, LA end-diastolic diameter and pulmonary artery systolic pressure have been found to be significantly high in terms of statistics (p<0.001). The comparisons of echocardiographic parameters are shown in table 4.

DISCUSSION

Reactive oxygen species (ROS) are produced in metabolic and physiological processes, and harmful oxidative reactions may occur in organisms. These harmful products removed by enzymatic and non-enzymatic antioxidative mechanisms. Antioxidant systems normally work in unity; they protect the cells from the toxic oxygen free radicals damage. Antioxidant molecules generated in the body prevents harmful substances by inhibiting them. Under certain conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the oxidative/antioxidative balance shifts towards the oxidative status. Consequently, oxidative stress, which has been implicated in over 100 disorders, develops (3).

In recent years, many diseases (cancer, coronary artery disease, chronic inflammatory diseases) are stated to be linked with increased free radical activity. Because of that oxidant / antioxidant balance is very important.

The mitral valve (MV) is a highly complex cardiac valve consisting of an annulus, anterior and posterior leaflets, chordae tendinea (chords) and two papillary muscles. The chordae tendinea mechanics play a pivotal role in proper MV function. There are many factors associated with chorda tendinea rupture. Mitral valve prolapse (MVP), rheumatic valve disease, calcific-degenerative valve disease, connective tissue diseases, cardiac trauma, hypertrophic cardiomyopathy, ischemic heart diseases and infective endocarditis (IE) have been reported as the causes of chorda tendinea rupture. Also pregnancy, hypertension and thalassemia have been reported as predisposing factors. (2)

Juang et al. analyzed 494 patients with ruptured chorda tendinea. Most of the patients (71%) were idiopathic, while remaining 29% had secondary causes. Of these 143 patients; 50 patients had subacute bacterial endocarditis, 35 patients had rheumatic heart disease, 61 patients had MVP, while 3 patients had other reasons (8). In our study; most of the cases of chordae tendinea rupture developed on rheumatic ground. Sixteen (53.3%) cases of chorda tendinea rupture occurred in the rheumatic mitral valve, 7 (23.3%) in degenerative valves and 7 (23.3%) in MVP.

Table 4. Comparison of echocardiographic parameters

	Chordae rupture (n=30)	Rheumatic MR (n=30)	Control (n=20)	p
Ejection fraction (%)	56.76±7.69	56.80±7.77	61.25±6.67	0.076
Left ventricle end–diastolic volume (ml)	94.43±21.89 *,#	110.00±25.96 **	69.85±12.21	<0.001
Left ventricle end–systolic volume (ml)	40.20±13.28 *	47.26±13.71 **	27.45±7.30	<0.001
Left ventricle end–diastolic diameter (mm)	54.40±4.93 *	54.76±4.71 **	45.85±1.49	<0.001
Left atrium diastolic volume (ml)	73.73±27.69 *	79.06±36.59 **	32.40±6.73	<0.001
Left atrium systolic volume (ml)	39.66±23.48 *	51.76±35.41 **	15.30±3.82	<0.001
Left atrium end–diastolic diameter (mm)	45.00±6.07 *	46.76±6.85 **	33.10±2.04	<0.001
Pulmonary artery systolic pressure (mmHg)	38.00±10.27 *	40.66±17.31 **	20.60±2.06	<0.001

Abbreviations: MR: Mitral regurgitation
*p<0.05 for chorda rupture vs control group
#p<0.05 for chorda rupture vs rheumatic MR group
**p<0.05 for rheumatic MR vs control group

In literature, oxidative stress is associated with cardiovascular risk factors including hypertension, endothelial dysfunction, increased systemic arterial stiffness, increased carotid wall thickness(9). As a result of recent studies, inflammation and oxidative stress may be a predisposing factor in the development of the chorda tendinea rupture (10).

Niao et al. reported that new evidence of increased oxidative stress in human severe mitral regurgitation, probably contributing to atrial enlargement. The serum oxidative stress index was significantly higher in the mitral regurgitation AF group and sinus group than in the lone AF group and healthy subjects (p<0.0001). Left atrial size was significantly larger in the mitral regurgitation AF group and sinus group than in the lone AF group and healthy subjects (p<0.0001). The oxidative stress index significantly and positively correlated with left atrial size in the overall study population (p=0.0008)(11).

Another study; Lloyd S.G et al. reported that myofibrillar degeneration can occur as a result of increased oxidative stress and hold responsible for the increase in the heart failure(12).

Previous studies have widely addressed the problem of oxidative stress in atherosclerosis and coronary artery disease, suggesting that oxidative stress might even be considered as a unifying mechanism for many cardiovascular risk factors. That vicious circle between oxidative stress and inflammation can occur not only in the diseased arterial wall, where it also causes loss of antioxidant protection and cell death (13). A recent pilot study assessing a limited number of patients suggested a possible link between serum oxidative stress index, left atrial enlargement and atrial fibrillation (14). All these results suggest that the oxidative stress may play a role in heart-valve pathogenesis.

Serum paraoxonase-1 activity is reduced in patients with heart valve diseases, caused by elevated oxidative stress and disturbances of heart valve metabolism. The findings from this novel detailed approach, implicate an inflammatory/oxidative stress process in the pathogenesis of the valve's presentation associated with the heart valve disease. The strength of the significance in differences encourage us to propose that the role of oxidative stress in heart valve disease pathogenesis is very prominent, and oxidative stress markers are potential ancillary tests to evaluate the state of the disease (15).

Also in other studies, oxidative stress has been regarded as one of the most important contributors to the progression of rheumatic and degenerative valve diseases (16, 17). Aydemir et al. reported that there were positive significant correlations between midkine, and reduced glutathione and selenium levels in patients with chorda tendinea rupture. According to their data in which selenium, zinc, midkine, and reduced glutathione decreased in chorda tendinea rupture patients, inflammatory response, oxidative stress, and trace element levels may contribute to etiopathogenesis of mitral regurgitation and/or ruptured chordae tendinea (10). Oxidative stress leads to vascular damage and participates in the pathomechanisms of aortic dissection and aneurysm formation. This study suggests that increased oxidative stress may play an important role in the thoracic aorta dissection (18).

In our study, oxidative/antioxidative balance is evaluated in all groups. Compared with the other groups, the TOA and OSI levels in the chordae rupture (group 1) have been found to be significantly high in terms of statistics. According to the results of our study, high oxidative stress might be accepted as a risk factor for chordae rupture.

Inflammation is an important contributor to the pathogenesis of rheumatic heart disease (RHD). It is a disorder of heart valves caused by a combination of immune, genetic and environmental factors. Cytokines are important mediators of inflammatory and immune responses. Cytokines are known to play an important role in regulating immunological and inflammatory reactions.

The cytokines TNF-α and IL-6 have an active role in the pathogenesis of many diseases. In the literature, cytokines play role in developing rheumatic valve disease, hypertension, coronary artery disease, heart failure and pulmonary hypertension (19,20,21).

TNF-α is a cytokine that has an active role in the pathogenesis of rheumatic diseases. Increased TNF-α levels have been shown when the heart is infiltrated by inflammatory cells (22). Davutoğlu et al. reported that plasma levels of IL-6, Interleukin-8(IL-8), IL-2 receptor (IL-2R), TNF-α and high-sensitive C-reactive protein (hs-CRP) were significantly higher in patients with RVD than in controls ($p < 0.001$). The chronic phase of RVD is associated with ongoing serum inflammatory mediators which correlate strongly with the severity of valve involvement, valve scarring, subsequent valve calcification and decreasing functional status. Future research in this area should focus on whether anti-inflammatory drugs might reduce progression, morbidity and mortality in patients with chronic RVD (23).

Mohamed et al. reported that TNF-α level was significantly higher in patients with rheumatic valvular involvement (24). Similar findings were found in the study by Rehman et al (25). Our findings were consistent with these two studies. TNF- alpha levels may be an indicator of increased inflammation in rheumatic mitral valve disease. This data supports that inflammation may be an important factor in the development of rheumatic valve diseases.

CONCLUSION

We showed that there is a significant relationship between chordae rupture and oxidative stress markers (TOA-OSI) in our study. High oxidative stress might be accepted as a risk factor for chordae rupture. In addition, TNF-alpha levels in the rheumatic severe mitral insufficiency group were higher than the chordae rupture group. These data support the role of inflammation in the developing of rheumatic severe mitral insufficiency.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gaziantep University Medicine Faculty.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors have no conflicts of interest to declare.

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Original Research

Effects of Two Decurarization Methods on Thermoregulation of Patients Under General Anesthesia

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ABSTRACT

Objective: Evaluate the effects of two decurarization methods on thermoregulation of patients who received general anesthesia.

Method: Sixty-six male patients that are over 18 years of age with an ASA physical status I-III and scheduled for elective thoracotomy. Patients were warmed with (38 ° C at medium setting) air blowing blankets. Decurarization was achieved with 0.02-0.05 mg/kg neostigmine in group N and 3 mg/kg sugammadex in group S after train of four (TOF) value obtained at the end of the operation.

Results: Core temperatures of tympanic membrane and peripheral skin temperatures of sternum and 1/3 upper part of the arm were measured and recorded at 15th, 30th, 45th, 60th, 90th and 120th minutes after extubation. Core temperature of the tympanic membrane and skin temperatures measured at the chest and arm in the first two hours after extubation were similar between the groups.

Conclusions: Decurarization agents had no effect on spontaneous central and skin temperatures in the actively heated postoperative patients that had previously equal intraoperative temperatures. Whereas the temperatures of the peripheral skin surface of the patients who received sugammadex returned to preoperative control levels earlier than that of patients receiving neostigmine.

Keywords: decurarization, hypothermia, neostgmin, sugammadex, thermoregulation

Body temperature is strictly controlled by multiple mechanisms under normal conditions (1). Threshold for cold response is expected to increase ten-fold under general anesthesia (2) and this increment effects heat control after the anesthesia. Temperature fluctuations after general anesthesia is stimulated by agents that are used in induction and maintenance of general anesthesia. Intraoperative hypothermia is associated with postoperative myocardial ischemia, cathecolamine levels and postoperative infections. Also, perioperative hypothermia has some adverse effects such as tremor, sedation, bleeding, increase in mortality and morbidity (3,4).

Unavoidable hypothermia is the most frequent thermal disorder during anesthesia. A combination of thermoregulation defect due to anesthesia and cold exposure in operating room results in hypothermia. Furthermore, about 20% of patients are involuntarily exposed to hypothermia that central body temperature is under 36 ° C in the preoperative period (3,5).

There are numerous methods that can be used in intraoperative and postoperative period to avoid hypothermia. Heating and humidifying of inhaled gases, heating of intravenously administered fluids and blood transfusion products, activating heat production of the patients by increasing the rate of metabolism are the methods that have been studied in the last decade (4,5).

Active and passive heat preservation methods are classified as internal and external heating methods. There is a growing concern regarding importance of temperature management (6,7).

The effects of anesthetical techniques and agents used for the purpose of induction and maintenance of anesthesia on thermoregulation (8) have been investigated in previous studies. Although the effect of reversed muscle strength on thermoregulation is known, MEDLINE survey has showed that the effects of decurarization methods on postoperative temperature have not been studied untill March 2015.

In recent years sugammadex, an aminosteroid agent, is also used in addition to the traditional decurarization agent neostigmine

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(9). In this study it is aimed to compare the effects of decurarisation methods on thermoregulation.

MATERIAL AND METHODS

Sixty-six male patients that are over 18 years of age with an American Society of Anesthesiologists (ASA) physical status I-III and scheduled for elective thoracotomy were enrolled in the study. The study was approved by the ethics committee (no: 2015-184, date: 15.06.2015), and voluntary approvals were received from the patients. Oral and written consents were obtained from the patients. Temperature of operation room was set at a standart of 22 °C.

Patients who had diabetes, coronary artery disease, thermoregulation impairing drug usage (i.e. beta blocker, calcium channel blocker, clonidine, steroids, antiepileptics, benzodiazepines), inflammatory disease, neuromuscular disease or dystrophic disorder, hypo-hyperthyroidism, Parkinson's disease, connective tissue disease (Raynaud syndrome etc.) and BMI (body mass ındex) scores over 30 kg/m2 or under 20 kg/m2 and also patients who remained intubated after the operation were excluded from the study. The existence of any life threatening clinical conditions (such as cardiac arrest) was accepted as a termination criteria for the study.

Preoperative body weight and height of the patients were recorded. Standart measures of vital findings, heart beat rate, noninvasive systolic and diastolic blood pressures, peripheral oxygen saturation (SpO2), body temperature (core temperatures of tympanic membrane and peripheral skin temperatures of sternum and 1/3 upper part of the arm) of the patients on each group were recorded before induction of general anesthesia. After preoxygenation with 3 L/min 100% O_2 for 3 min before anesthesia, induction of general anesthesia was provided intravenously by 1 µg/kg fentanyl, 1-2 mg/kg propofol, 0,5 mg/kg rocuronium. Patients were mechanically ventilated after entubation. Infusion of 5-6% desflurane, 0.1-0.2 mg/kg rocuronium iv bolus and 0.05-0.1 µg /kg/hour remifentanyl were used for maintenance. Fresh gas flow was kept as 2 L/min during the operation. Subclavian vein catheterization was performed for each patient. The crystalloid and colloid solutions were kept in room temperature for 24 hours. After the patients were covered with surgical drapes, they were heated with forced air warmer (Tyco Healthcare Group LP Nellcor Puritan Bennett Division Pleasanton, CA U.S.A. 1-800-NELLCOR) in medium (38 °C) settings. The durations of staying in the opreting room of each patient were recorded.

Closed envelope method was performed for randomization. In the period of skin closure the envelope was opened. The patients were classified into 2 groups;

Group N was composed of the patients who were decurarised with Neostigmin atropin (N=33) and Group S was composed of the patients who were decurarised with Sugammadex(N=33).

Decurarisation was performed with 0,02-0,05 mg/kg iv. Neostigmin and 0,01-0,02 mg/kg iv. atropin in neostigmin group (GROUP N) and with 3 mg/kg iv. Sugammadex in Sugammadex group (GRUP S) when TOF (train of for) value could be obtained after the operation was completed and by the time the administration of inhalation anesthetic was ceased. The patients who had TOF of 90% under neuromuscular monitorization were extubated.

All patients were directly transfered to postoperative intensive care unit after the operation. Patients were covered with a single layer of cotton blanket. As the patients were transfered to intensive care unit, core temperatures of tympanic membrane and peripheral skin temperatures of sternum and 1/3 part of the upper arm were measured and recorded at the 15th, 30th, 45th, 60th, 90th and 120th minutes after extubation. These measurements were performed and recorded by experienced intensive care unit nurses. Tympanic membrane core temperatures were measured by using "Braun thermoscan designed in Germany made in Mexico". Skin temperatures were measured with HT-F03B infrared thermometer.

Statistical Analysis

The sample size of patients per each group was calculated using G power analysis. To accept 25% difference between groups, by means of returning to preoperative temperatures, as statistically significant; the minimum total number of patients required was found to be 64. (α= 0.05 β= 0.95)

Shaphiro wilk test was used to find out whether numerical data were normally distributed or not. Means of independent samples were compared by using Student t test. Two way analysis of variance with repeated measures were used to compare groups at various times. Chi square test was used to test the correlation between cathegorical variables and to test the correlation between numerical variables Correlation analysis was used. Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 22.0 was used for the analysis. A p value below 0,05 was accepted as statistically significant.

RESULTS

A total of 71 patients were enrolled to the study. But five patients were excluded due to failure of extubation in the operating room in four patients and 1 patient was reoperated in 2 hours because of surgical bleeding.

No statistically significant difference was present between 2 groups in terms of age, ASA, BMI, and duration of operation ($p>0.05$) (Table 1). Comparison of the groups in terms of preoperative skin temperatures (chest and arm) and core temperatures of tympanic membrane revealed that the difference was statistically insignificant. ($p> 0.05$) (Figure 1).

No statistically significant difference was found between the groups in terms of tympanic temperatures that were recorded at the 15th, 30th, 45th, 60th, 90th and120th minutes after extubation (Figure 2) ($p > 0.05$) . In each groups, tympanic temperatures that were measured in 15th, 30th, and 45th minutes of postoperative period were significantly lower than preoperative control values ($p < 0.05$) (Figure 2) . No statistically significat difference was found between the groups in terms of the time passsed for returning to preoperative tympanic temperature (p

Table 1. Demografic Values

Group	Age (year)	ASA	Operation Time (Min)	BMI
GROUP N	54.09±16.7	2.45±0.56	138.48±29.88	25.63±2.66
GROUP S	51.24±18.07	2.48±0.61	139.39±31.11	25.66±2.67

BMI: Body Mass İndex

> 0.05) (Figure 3). Skin temperatures of chest in 15th, 30th, 45th, 60th, 90th, 120th minutes after extubation were statistically similar betweeen the groups ($p > 0.05$) (Figure 4) .

In both groups skin temperatures of chest that were recorded at 15th, 30th and 45th minutes of postoperative period were significantly lower than preoperative control values ($p < 0.05$).

No statistically significantly differences were found between the groups in terms of skin temperatures of arm that was recorded at 15th, 30th, 45th, 60th, 90th, and 120th minutes after extubation ($p > 0.05$) (Figure 5). However, skin temperatures of arm that was measured at 15th, 30th and 45th minutes of postoperative period were significantly lower than preoperative control values in Group N. ($p < 0.05$) . On the contrary, skin temperatures of arm that were measured at 15th and 30th minutes of postoperative period were significantly lower than preoperative control values in Group S ($p < 0.05$) .

DISCUSSION

General anesthetics, especially when used in long lasting surgical procedures, compromise thermoregulation mechanisms (10,11). Therefore, male patients who had thoracotomy operation with long lasting anesthesia and who were transfered to postoperative intensive care unit were preferred for this study. Just because progesteron is known to compromise thermoregulation in female population, male gender was preferred in the study to prevent physiological bias (12, 13).

Jung KT et al. (14) compared inhalation anesthesia with desflurane and TIVA (propofol, remifentanil) in patients who had tympanoplasty. Postoperative core temperatures of the patients that TIVA was administered was reported significantly higher than those taking desflurane, on the other hand it was determined that peripheral thermoregulatory vasoconstruction occured earlier and more frequently in TIVA taking group. In their study, patients were not heated actively but in our study the patients were actively heated during intraoperative period. Absence of significant difference in postoperative central and skin temperatures would proceed from this situation. Intragroup analysis revealed that postoperative tympanic core temperatures reached up to preoperative tympanic temperature values statistically at the 60th minute in both groups. Postoperative temperatures of arm skin that were recorded at 15th, 30th and 45th minutes were found significantly lower than control values of preoperative period ($p < 0.05$) in Group N. On the other hand, postoperative temperatures of arm skin in Group S at 15th and 30th minutes were significantly lower than control values of preoperative period ($p < 0.05$). Postoperative skin temperatures that were measured from arm region in Sugammadex group had reached up to preoperative temperature 15 minutes earlier. This would be due to two situations; first of all it is possible that sugammadex might have caused more peripheral thermoregulatory vasoconstriction or secondarily the patients in Group S would have achived peripheral heat control more rapidly due to having better muscle function reversal.

Anticholinergic agents decrease heat loss by lessening perspiration and causes an increment in central temperature. Furthermore, it is suggested that atropine leads to temperature rise by central mechanisms (15, 16). Mirakhur and Dundee (17) have shown that atropine and glycopyrrolate, in clinical doses, would result in 0.3 °C increase in oral temperatures in resting state and while exercising. Simpson et al. (18) determined that anticholinergic agents could rise the central temperature in resting and exercising but if this was compared to saline administered group, there was no statistically significant difference. In our study 0.02-0.05 mg/kg iv. Neostigmine with 0.01-0.02 mg/kg iv. Atropine were used. Therefore, rather than assessing efficiency of neostigmine on thermoregulation; our measurements have shown the efficiency of neostigmine/ atropine combination that is used traditionally in clinical practice.

In the study of Frank et al. (19), 44 patients who had radical prostatectomy under spinal anesthesia and who were not actively heated intraoperatively and postoperatively were given warmed blood products and intravenous fluids and tympanic core temperatures were measured. age, lipid rate and spinal anesthesia level were found correlated with hypothermia formation. Age and spinal anesthesia level were found correlated with hypothermia when the patients were compared in terms of age, spinal blockage level, operating room temperature, and body form (body fat ratio, BMI). Every increase in age leads up to 0.3 °C loss in central temperature. In our study, the patients were between the ages of 18- 77. There was no significant correlation between age and postoperative tympanic temperatures irrespective of the groups.

One of the limiting factors in our study was wide distribution of age. In our study tympanic temperatures of the patients were assessed at postoperative 180th minute. Tympanic temperatures in postoperative 180 th minute were found lower than preoperative temperatures. In our study, the patients were followed up to postoperative 120th minute. All patients have returned to their preoperative tympanic temperatures in 120th minute of the postoperative period. This difference would come from the fact that we actively heated the patients intraoperatively.

In the study that Washington et al. (20) applied anesthesia to healty volunteers, in two different days. The volunteers in similar age, body lipid rate, weight and height were enrolled to this study. Enflurane and nitrous oxide were used for anesthesia and vecuronium was used for muscle relaxation. Patients were actively warmed with forced air during the anesthesia and were given electrical pain stimulation. Thermoregulatory vasoconstriction was defined as temperature difference between forearm and fingertip that exceeds 4 ° C. Thermoregulatory vasoconstriction treshold was found significantly higher when painful stimulus was given. Therefore, it seemed that intraoperative or postoperative pain control lowers thermoregulatory vasoconstriction treshold and by this way increases heat loss. Another limitation of our study may be that we did not evaluate the postoperative pain scores of the patients.

In conclusion; decurarisation agents did not have any efects on spontaneous postoperative central and skin temperatues of the patients that were actively warmed in equal intraoperative heat conditions but peripheral temperatures measured from arm skin of the patients that used sugammadex reached up to preoperative control levels more rapidly. Only 15 minutes earlier recovery of peripheral temperatures without any changes in central temperatures would partially reflect that successful recurarization improves thermoregulation. Neverthless, there is need for further studies that will be planned without applying active heating in age matched groups.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gaziantep University (no: 2015-184/15.06.2015).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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Original Research

Outcome of Elderly Nasopharyngeal Carcinoma Patients: A Single Center Study

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ABSTRACT

Objective: This study aimed to assess the efficiency of radiotherapy and evaluate its outcomes for elderly (>65 years) patients who have undergone treatment for nasopharyngeal carcinoma (NPC).

Methods: Forty-five (male, 35; female, 10) elderly patients with a diagnosis of undifferentiated NPC who were treated at our institution between 1994 and 2012 were retrospectively evaluated. The primary endpoint was the relationship between the patients' characteristics and overall survival (OS); progression-free survival (PFS), locoregional progression-free survival (LR-PFS), and toxicity analysis were the secondary endpoints.

Results: The patients had a median age of 74.2 years. At a median follow-up period of 64 months, the median OS, PFS, and LR-PFS were 45 (95% confidence interval [CI]: 5.887-84.113), 34 (95% CI: 0.0-70.504), and 45 (95% CI: 20.092-69908) months, respectively. The 2-, 3-, and 5-year OS rates were 61.5%, 53.1%, and 50.0%, respectively, and the 2-, 3-, and 5-year PFS rates were 57.6%, 46.8%, and 43.7%, respectively. Patients with T stage (T3-T4 vs.T1–T2) or N stage (N0–1 vs. N2) had significantly shorter OS ($p<0.05$), PFS ($p<0.05$), and LR-PFS ($p<0.05$) outcomes, respectively, which were also confirmed using a multivariate analysis ($p<0.05$).

Conclusion: Our results demonstrated that the established prognostic factors, including T and N stages, were important prognostic indicators of NPC in elderly patients.

Keywords: Chemoradiotherapy, elderly, nasopharyngeal carcinoma, survival

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial malignant tumor of the nasopharynx (1). However, squamous cell carcinoma is the most common histopathological type of NPC (2). The incidence rate ranges from 20 to 30 per 100.000 individuals (3). Although the early detection of NPC is important for its curability and for reducing treatment-related toxicity, majority of the patients present with symptoms of an advanced state of NPC (4).

Based on the location of the nasopharynx and due to the high radiosensitivity of the disease, radiotherapy is the standard management strategy for nonmetastatic disease (5, 6). However, the age and comorbid conditions of elderly patients with NPC pose a unique challenge for radical treatment (7). Intensity-modulated radiotherapy (IMRT), which has an advantage of more precise coverage using sharp-dose gradients, has been accepted as the gold standard radiotherapy technique that may improve tumor control and quality of life (8-10). Unfortunately, there are limited oncology centers that follow the modality of employing three-dimensional conformal radiation therapy (3D-CRT) and brachytherapy as a boost to the dose supplement.

Therefore, this study aimed to evaluate the clinical outcomes of NPC in elderly patients undergoing 3D-CRT and brachytherapy (BRT) as the treatment boost.

METHODS

A total of 45 elderly patients with NPC, treated between June 1994 and June 2012, Department of Radiation Oncology, were

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retrospectively analyzed. The median follow-up duration was 32 months (range: 6-193 months). Although no defined age cutoff currently exists for patients in the field of oncology, the patients in this study were defined as elderly if they were aged ≥65 years. The patients included in this study were selected from 558 patients who underwent definitive radiation therapy (RT) or chemoradiation therapy (CRT).

Clinical staging was performed using head, neck, and thoracic magnetic resonance imaging (MRI), abdominal computed tomography (CT), and whole-body bone scanning. In the past decade, the clinical staging of most patients with distant metastasis was performed using 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography. All the patients were staged according to the American Joint Cancer Committee (AJCC 7th edition, 2010) TNM staging system guidelines.

Ethical Considerations

All the procedures were performed in accordance with the ethical guidelines of the institutional and/or national research committee and with the guidelines of 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all the participants included in the study.

Treatments

All the patients were treated daily with doses of 1.8-2.12 Gy (median: 2 Gy). Every patient received 60-70 Gy at the primary tumor site. Patients who underwent CRT were treated with the most frequently used concurrent single agent, which was a platin-based (cisplatin or carboplatin) agent. Cisplatin (25-40 mg/week) was concomitantly admininstered for 7 weeks. Furthermore, during the course of the treatment, RT was administered using two techniques: conventional RT and 3D-CRT. BRT, in combination with external RT, was used to boost the dose supplementation in patients with locally persistent tumors. Subsequently, intracavitary BRT was administered at a total dose of 12 Gy in three fractions after external RT. BRT was performed using a high-dose rate MicroSelectron device (Netherlands), an Ir-192 source, and special single-channel applicators.

Statistical Analysis

The primary focus was to investigate the effect of potential prognostic factors associated with OS, while the secondary focus was to evaluate the associations between the prognostic factors, PFS, and LR-PFS. Descriptive statistics and the Kaplan-Meier survival test were performed to evaluate the population frequencies and estimate the overall survival (OS), respectively. OS was defined as the interval between the diagnosis date and death/last follow-up date. The interval between the first day of concomitant CRT and progression/recurrence at the nasopharynx and/or ipsi-/contralateral neck or death/last follow-up date (for LR-PFS), and any disease progression or death/last follow-up date (for PFS), respectively, were estimated. The data were analyzed using Statistical Package for the Social Sciences v.22.0 for Windows (IBM Corp., Armonk, NY, USA). Categorical variables were expressed as frequency, whereas numerical variables were expressed as descriptives. $p<0.05$ was considered significant.

RESULTS

The median age of the patients was 74.2 years (range: 65–82 years). Of the 45 patients in total, 35 (77.7%) were males and 10 (22.3%) were females. The distribution of the patients who were treated according to the AJCC 2010 staging system during the diagnosis is as follows: T1 (n=14; 31.2%), T2 (n=8; 17.6%), T3 (n=14; 31.2%), T4 (n=9; 20%), N0 (n=20; 44; 5%), N1 (n=14; 31.1%), and N2 (n=11; 24.4%). A diagnosis analysis showed that 7 patients (15.6%) had stage I, 8 (17.6%) had stage II, 21 (46.8%) had stage III, and 9 (20%) had stage IVA NPC.

All the patients were treated with doses of 1.8–2.12 Gy (median: 2 Gy) per day. The primary tumor site received a dose of 60–70 Gy (median: 70 Gy). The following treatments were administered: RT alone, n=16 (35.5%); concurrent CRT, n=12 (26.7%); CRT+adjuvant chemotherapy (CT), n=3 (6.7%); neoadjuvant CT+CRT, n=8 (17.7%); and neoadjuvant CT+RT, n=6 (13.4%). The median RT duration was 52 days (range: 30–73 days). Of all the patients, 26 (57.7%) received BRT as a treatment boost. The patient characteristics and treatment details are listed in Table 1.

The last follow-up visit indicated that 17 (37.7%) patients survived with no evidence of the disease, 2 (4.5%) survived with local recurrence (LR), and 1 (2.2%) survived with distant metastasis. Furthermore, 25 (55.6 %) patients died, of which 20 (43.5%) deaths were caused by disease recurrence, 2 (4.4%) by treatment-related toxicity, and 3 (6.6%) by nontumor-related causes. The 2-, 3-, and 5-year OS rates were 61.5%, 53.1%, and 50.0%, respectively, and the median OS time was 45 months (95% CI 5.887–84.113). The evaluation of the patient survival rates was performed using a univariate analysis based on the T stage (1–2 vs. 3–4), N stage (0–1 vs. 2), sex, treatment modality, BRT boost, and age groups (<70 years vs. ≥70 years). The estimated OS for the T stage 1–2- and 3–4-group was 95 and 22 months, respectively, which was statistically significant (p=0.003). However, the other potential prognostic factors (age, treatment modality, and BRT boost) did not show any effect on OS. The results of the univariate analysis revealed that a lower T stage (1–2 vs. 3–4) and N stage (0–1 vs. 2) was associated with a significantly improved OS rate (Figure 1). However, in the multivariate analysis, these factors remained independent of the OS rate.

The 2-, 3-, and 5-year PFS rates were 57.6%, 46.8%, and 43.7%, respectively, and the median PFS time was 34 months (95% CI: 0.0–70.504). The estimated PFS for the T stage 1–2- and 3–4-group was 83 and 18 months, respectively, which was statistically significant (p=0.002). Furthermore, the estimated PFS for the N stage 0–1- and 2-group was 34 and 11 months, respectively, which was statistically significant (p=0.009). However, the other potential prognostic factors showed no effect on PFS. The outcomes of the univariate analysis revealed that a lower T stage (1–2 vs. 3–4) and N stage (0–1 vs. 2) was significantly associated with better PFS outcomes (Figure 2). However, the results of the multivariate analysis revealed that these factors remained independent of the PFS rate.

Table 1. Patients characteristics and treatment details in patients with elderly NPC

Characteristics	Patients	
	Number	Percent
Gender		
Male	35	77.7
Female	10	22.3
Age (year)		
<70	21	46.7
≥70	24	53.3
Brachytherapy boost		
Present	26	57.7
Absent	19	42.3
WHO Morphology		
type I (keratinizing)	3	6.7
type II(non–keratinizing)	25	55.5
type III (undifferentiated)	17	37.8
External Radiotherapy dose		
<6500 cGy	6	13.3
≥6500 cGy	39	86.7
T stage (2010 AJCC 7th)		
T1	14	31.2
T2	8	17.6
T3	14	31.2
T4	9	20.0
N stage (2010 AJCC 7th)		
N0	20	44.5
N1	14	31.1
N2	11	24.4
TNM stage (2010 AJCC 7th)		
I	7	15.6
II	8	17.6
III	21	46.8
IV a	9	20.0
Treatment Modality		
RT	16	35.5
CRT	12	26.7
CRT+Adjuvant CT	3	6.7
Neoadjuvant CT+ CRT	8	17.8
Neoadjuvant CT+RT	6	13.3
CT		
Yes	17	37.8
No	28	62.2
Concomitant CRT		
Yes	23	51.1
No	22	48.9

AJCC: American Joint Cancer Committee; RT: Radiotherapy; CRT: Chemo–radiotherapy; CT: Chemotherapy

During the follow-up period, 10 (22.3%) patients experienced recurrences, of which 7, 2, and 1 experienced local, regional, and locoregional recurrences, respectively. The 2-, 3-, and 5-year LR-PFS rates were 67.5%, 53.1%, and 50.0%, respectively. The median LR-PFS time was 45 months (95% CI: 20.092–69908). The estimated LR-PFS for the T stage 1–2- and 3–4-group was 83 and 18 months, respectively, which was statistically significant (p=0.001). Additionally, the estimated LR-PFS and for the N stage 0–1- and 2-group was 62 and 25 months, respectively, which was statistically significant (p=0.034). The results of the univariate analysis revealed that a lower T stage (1–2 vs. 3–4) and N stage (0–1 vs. 2) were significantly associated with an improved LR-PFS (Figure 3). The results of the univariate analysis are summarized in Table 2. In contrast, the multivariate analysis showed that these factors remained independent of the LR-PFS rate; the results of the multivariate analysis are summarized in Table 3.

Toxicity

Xerostomia (Grade≤2) was the most frequent treatment-related complication that was reported in 31 (68.9%) patients. During the follow-up, late severe complications were observed in 9 (20%) patients, of which 5 (11.1%) had hearing loss and 4 (8.9%) had optic neuropathy. Other toxicities observed in the patients included neck fibrosis (2.2%), Lhermitte's sign (2.2%), brain necrosis (2.2%), and bleeding (2.2%); the details of these complications are summarized in Table 4.

DISCUSSION

In this study, the poor OS, PFS, and LR-PFS outcomes in elderly patients with advanced T and N stages of NPC clearly indicate that these established parameters are important prognostic indicators in elderly patients.

A study conducted by Xiao et al. (11) on patients with early-stage NPC demonstrated that although the 5-year OS rates in T1N0, T2N0, and T1N1 were reported to be comparable, unfavorable OS outcomes were reported in patients with T2N1 when compared to patients in other groups. Moreover, our results, which are in line with the results of this study, reveal that elderly patients with higher T (T3–4 vs. T1–2) and N (N2 vs. N1–0) stages of NPC have lower OS (p=0.002), PFS (p=0.002), and LR-PFS (p<0.001) rates.

Based on the fact that older patients with NPC (>70 years) are usually excluded from clinical trials, the current management strategy for this group was performed according to guidelines for adult and/or studies including patients aged 60–65 years (12-14). However, patients with NPC, aged >70 years, are more likely to have various comorbidities and a poor performance status, limiting the efficacy of radiotherapy and chemotherapy and subsequently resulting in more unfavorable outcomes (7, 15-18). In an IMRT study including patients with NPC, aged >70 years, approximately 30% of the deaths were caused by internal medical problems that were not associated with the cancer. Furthermore, the 5-year OS rate has been shown to be significantly higher in patients with a good performance status (18). Therefore, future investigations should include more homogenous populations comprising older patients, which may help to update the current literature.

Figure 1. a, b. Overall Survival curve in 45 elderly patients with NPC. a. OS curve. a. Log-Rank curve of OS estimation for T status (p=0.003)

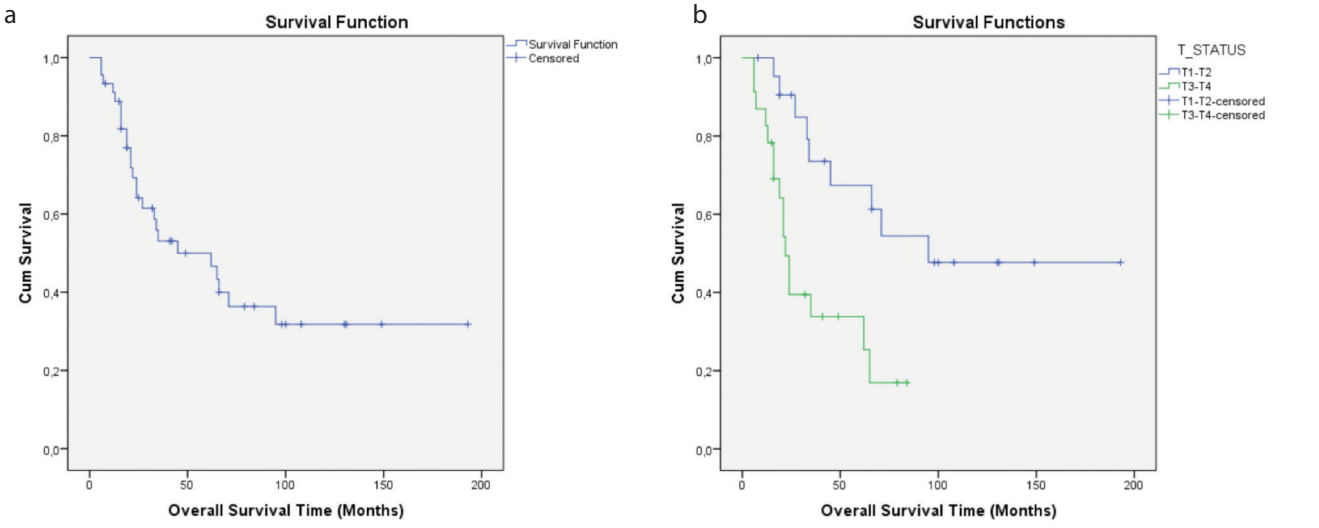


Figure 2. a-c. Progression Free Survival in 45 elderly patients with NPC. a. PFS curve. b. Log-Rank curve of PFS estimation for T status (P=0.002). c. Log-Rank curve of PFS estimation for N status (P=0.009).

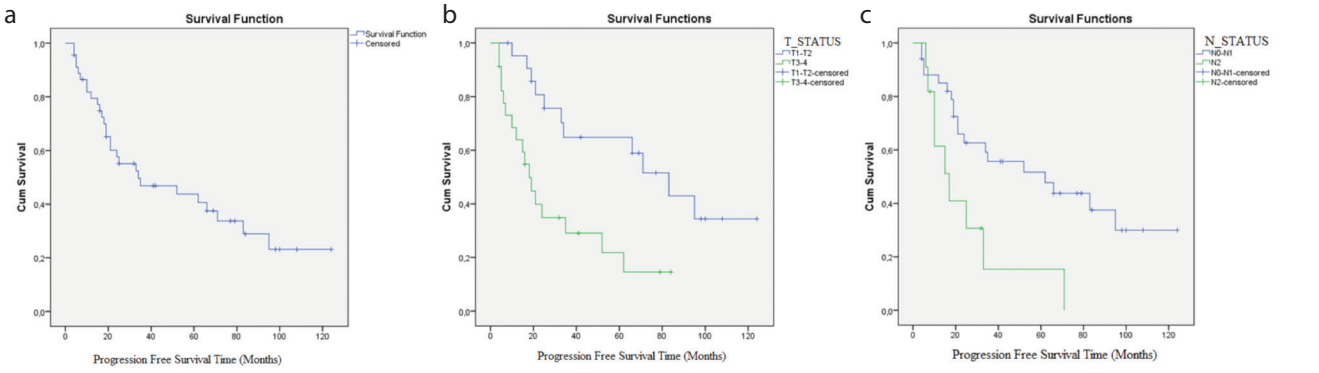
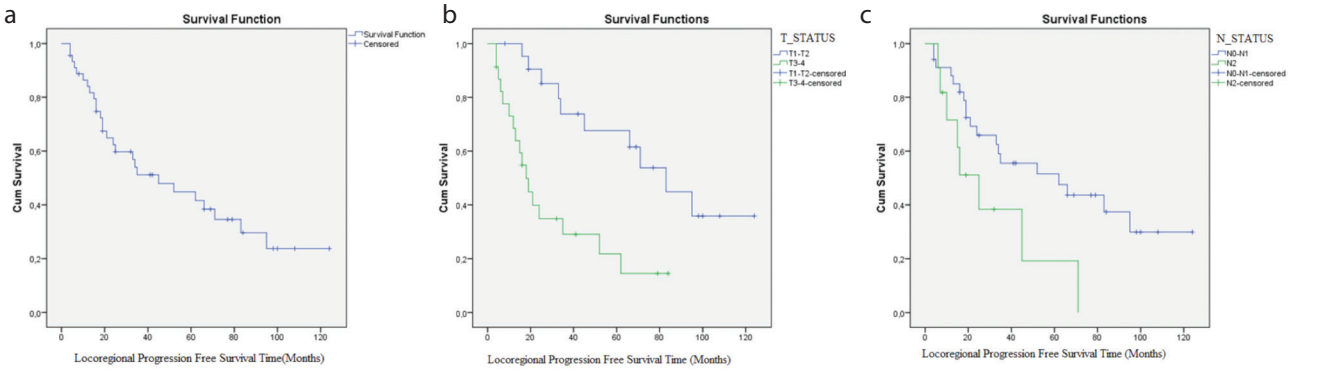


Figure 3. a-c. Locoregional -Progression Free Survival in 45 elderly patients with NPC. a. LR-PFS curve. a. Log-Rank curve of LR-PFS estimation for T status (p=0.001). c. Log-Rank curve of LR-PFS estimation for N status (P=0.034).



In contrast, no advantage of the multimodal treatment was observed in the elderly patients. Moreover, radiotherapy treatment might be a better option to avoid toxicity. Therefore, an improvement in radiotherapy and chemotherapy is thought to lead to the reduction of acute and late toxicity, thereby improving the quality of life of the patients.

Our study has several limitations. The first and major limitation of this study is its retrospective nature. Moreover, some patients were examined by CT scan of the nasopharynx and neck post 2010, rather than by MRI. Therefore, the patient staging may have been inaccurate. In this regard, additional

Table 2. Univariate analysis with Kaplan–Meier for prognostic factors in patients with elderly NPC

Analysis data	OS			PFS			LR–PFS		
	Survival Rates			Survival Rates			Survival Rates		
Variant	3–5 years (%)	Months (Median)	p	3–5 years (%)	Months (Median)	p	3–5 years (%)	Months (Months)	p
T stage(AJCC)									
T1–T2	73.5– 67.4	95	0.003	64.8–58.9	83	0.002	73.8–67.7	83	0.001
T3–T4	49.3–33.8	22		29.1–21.8	18		29.1–21.8	18	
N stage(AJCC)									
N0–N1	66.5–56.6	65	0.065	55.7–51.7	34	0.009	55.5–51.5	62	0.034
N2	42.1–21.0	27		40.9–15.3	11		51.1–19.2	25	
Age (years)									
<70	39.0–31.2	34	0.346	37.3–29.8	24	0.545	43.3–29.7	24	0.388
≥70	64.2–58.8	66		64.7–55.0	62		63.2–57.5	66	

OS: Overall Survival; PFS: Progression Free Survival; LR–PFS: Locoregional Progression Free Survival; AJCC: American Joint Cancer Committee

Table 3. Multivariate analysis with Cox Regression for prognostic factors in patients with elderly NPC

	OS			PFS			LR–PFS		
Variant		SE	p	P	SE	p	P	SE	p
T status (AJCC)									
(T1–T2 vs. T3–T4)	0.002	0.453	0.001	0.002	0.416	0.00	0.00	0.434	0.00
N status (AJCC)									
(N0–N1 vs. N2)	0.028	0.472		0.004	0.439		0.009	0.459	

OS: Overall Survival; PFS: Progression Free Survival; LR–PFS: Locoregional Progression Free Survival; SE: Standard Error; AJCC: American Joint Cancer Committee

Table 4. Late toxicities of subsequent radiotherapy

Complication	Number of patients	Percent
Xerostomia	31	68.9
Hearing loss	5	11.1
Optic neuropathy	4	8.9
Neck Fibrosis	1	2.3
Lhermitte's sign	1	2.3
Brain Necrosis	1	2.3
Bleeding	1	2.3
Total	44	98.1

prospective randomized clinical trials are needed to clearly determine the optimal treatment in elderly patients with NPC.

CONCLUSION

The present findings demonstrate the prognostic value of the established T and N stages in elderly patients with NPC.

Ethics Committee Approval: Authors declared that the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013)

Informed Consent: Written informed consent was obtained from participants who participated in this study.
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Original Research

Normal Main Portal Vein Diameter – Is the Upper Limit Of 13 Mm Low?

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ABSTRACT

Objective: We aimed to compare the normal main portal vein diameter measured in computed tomography with the commonly used upper limit value.

Materials and Method: Computed tomography examinations performed between March 2015 and April 2018 in our department were scanned from the archive system. Mean portal vein diameters were measured on axial contrast-enhanced and non-enhanced abdominal CT scans of the patients without any known disease.

Results: 500 main portal vein measurements were performed from 276 individuals. In the non-enhanced images (n = 243), the mean diameter of main portal vein was 15.03 ± 1.72 mm and in the post-contrast enhanced images (n = 257) the mean diameter of the main portal vein was 15,05 ± 1.71 mm. These values showed a significant difference from the widely accepted upper limit of 13 mm (95% confidence interval for non-enhanced images: 1.81-2.25 mm higher, p <0.001, 95% confidence interval for post-contrast images: 1.84-2.26 mm higher, p <0.001). The mean main portal vein diameter measured from contrast tomography images was 0.26 mm wider than the mean main portal vein diameter measured at non-enhanced images (95% confidence interval: 0.23-0.29 mm, p <0.001).

Conclusion: The mean normal portal vein diameter measured in computed tomography (15.05 mm) was significantly higher than the accepted upper limit of 13 mm (p <0.0001). The mean main portal vein diameter in contrast-enhanced tomography was 0.26 mm larger than the mean main portal vein diameter measured in the non-enhanced examination.

Keywords: Portal vein, mean diameter, upper limit, portal hypertension, computed tomography.

INTRODUCTION

Increased diameter of the portal vein is a finding of portal hypertension. There are studies which show the probability of increased esophageal variceal frequency as the portal vein diameter increases (1).

Ultrasound examination is an important diagnostic method for detecting portal hypertension (2-4), and most of the studies on portal vein diameter in the early studies were performed with ultrasound. The upper limit values for normal portal vein diameter reported in these studies which were performed with ultrasound, were found to be between 11.7 and 14 mm. Furthermore, commonly accepted literature indicate that the normal upper limit value of the main portal vein is 13 mm (8-10). However, when evaluating abdominal computed tomography (CT) examinations in our daily routine, we observed that the main portal vein diameter was higher than this commonly accepted value mentioned above in many CT examinations, and we thought that it was important to determine whether the upper limit value of the main portal vein diameter is larger than the previously mentioned values. In this study, we aimed to compare the normal main portal vein diameter measured by CT scans with the commonly used upper limit value.

MATERIALS AND METHODS

The study was conducted retrospectively with the approval of the Ethics Committee of our university (Decision No: 2018/245). In this retrospective study, computerized tomography examinations performed at the Department of Radiology, Medical School between March 2015 and April 2018, were scanned from the archive system and the main portal vein diameter measurements were obtained from non-contrast and contrast-enhanced tomography images of patients without any known disease. Patients with liver diseases or liver enzyme disorders were excluded from the study.

Imaging Technique

CT imaging was performed on a 64-section CT device (General Electric Lightspeed VCT-XTe; GE, Milwaukee, USA). The scan area

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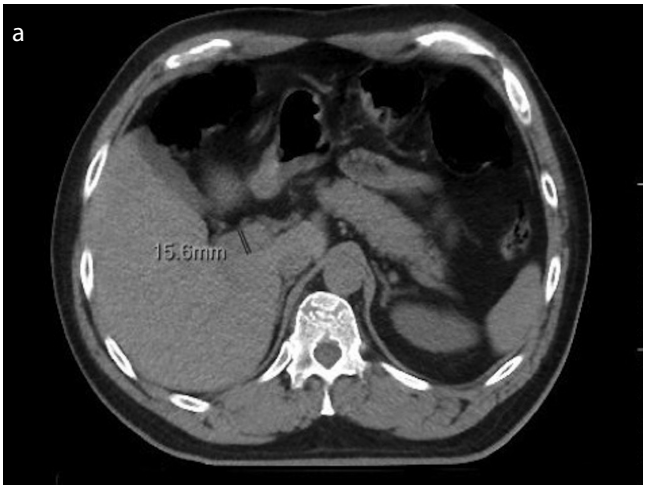
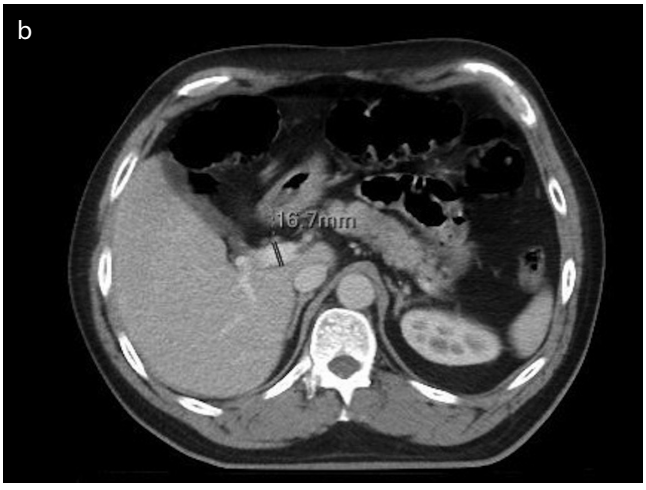
Table 1. Main portal vein diameter (mm) in non–contrast CT and minimum, maximum, mean and standard deviation values of main portal vein diameters (mm) in contrast–enhanced CT

	Number of patients (N)	Minimum Value	Maximum Value	Mean	Standard Deviation
Main portal vein diameter in non–contrast CT (mm)	243	10.45	22.33	15.0344	1.72951
Main portal vein diameter in contrast–enhanced CT (mm)	257	10.75	20.11	15.0512	1.71864

Figure 1. a, b. Change in low-density lipoprotein cholesterol levels with time and drug use in the presence of (a) HbA1c covariate and (b) body mass index covariate





was between the level of the diaphragm and the symphysis pubis. Shooting parameters 100 kV; 450etal; section thickness 5 mm; The gantry angle was 0 °. Contrast-enhanced intravenous contrast medium was given as 1.5–2 ml / kg.

Evaluation of Images

CT images were evaluated on the workstation via PACS (image archiving and communication system). Portal vein diameter measurements were performed on axial contrast-enhanced and non-enhanced abdominal CT scans. Measurements were made 1 cm distal from the junction of the splenic vein and superior mesenteric vein and at least 1 cm proximal without giving the first branch of the main portal vein. Measured values were recorded in milimeters.

Statistical analysis

The suitability of the data for normal distribution was tested with the Shaphiro wilk test. Student's t test was used for comparison of the normally distributed variables in two groups, and Mann Whitney u test was used for the non-normally distributed variables. Single sample t test was used to test the difference of numerical variables from a standard value, and paired t test was used to compare two dependent measurements. Spearman rank correlation coefficient was used to investigate the relationships between numerical variables. Descriptive statistics mean ± standard deviation for numerical variables, 95% confidence interval and number and% values for categorical variables. SPSS for Windows version 22.0 (Armonk, NY: IBM Corp) was used for statistical analysis and p <0.05 was considered statistically significant.

RESULTS

A total of 500 main portal veins were measured from a total of 276 individuals, 124 of which were female (55.1%) and 152 were male (44.9%). The mean age was 54.05 ± 14.8 years (range 21-88). The mean diameter of the main portal vein was 15.03 ± 1.72 mm in the non-enhanced examinations (n = 243), and 15.05 ± 1.71 mm in the mean examination in contrast-enhanced examinations (n = 257). This value was significantly different from the commonly accepted 13 mm (95% confidence interval for non-contrast CT: 1.81-2.25 mm higher, p <0.001; 95% confidence interval for contrast CT: 1.84-2.26) mm higher, p <0.001). The mean diameter of the main portal vein measured by contrast-enhanced tomography was 0.26 mm wider than the mean diameter of the main portal vein in non-enhanced examination (95% confidence interval: 0.23-0.29 mm, p <0.001). Table 1 shows the minimum, maximum, mean and standard deviation of the main portal vein diameter (mm) in non-enhanced CT and the main portal vein diameters (mm) in contrast-enhanced CT.

DISCUSSION

In this retrospective study, the mean mean portal vein diameter (15.05mm) measured on computed tomography was significantly higher than that of the commonly accepted upper normal limit, which is 13 mm. The mean main portal vein diameter measured from contrast-enhanced tomography was 0,26 mm wider than that of the non-contrast series.

In our study, the normal main portal vein diameter measured on CT examination was significantly different from the commonly

accepted 13 mm. Considering that we found the mean main portal vein diameter to be 15.5 mm in healthy subjects on CT, we expect that the upper normal value will be higher than this value. For this reason, it will be useful to perform CT studies to assess the predictive value between healthy and diseased people.

In the literature, there have been studies performed with ultrasound and found that the mean portal vein diameter was lower in healthy subjects (5-7).

In a retrospective study conducted by Stamm et al., who studied the mean portal vein diameter via CT evaluation similar to how it was performed in our study, the mean portal vein diameter was found to be 15.5 ± 1.9 mm in contrast and non-enhanced CT series of 191 healthy subjects, which is quite close to that of measured in our study, with the average value of 15.05 mm. In the present study, the mean diameter of the main portal vein measured by contrast-enhanced tomography was 0.26 mm wider than the mean diameter of the main portal vein in non-enhanced examination (95% confidence interval: 0.40-0.71 mm, p <0.0001). In our study, there was a difference between the mean portal vein diameters measured in non-enhanced and contrast-enhanced imaging, which was slightly lower than in the present study (1.84-2.26 mm p <0.001).

In general, during abdominal CT examination, the patients are asked to take deep breaths just before CT imaging begins, which is done so in our department as well, and CT scans are performed while the patient is still holding his or her breath. In studies conducted with ultrasound examination in the literature, it is noticed that in some of these studies, the measurements were taken during normal respiration (12) and in some other studies, the respiratory phase of the measurement of the patient was not specified (5, 7). The discrepancies of the values of mean portal vein diameter may be due to these different methodologies applied during ultrasound and CT examinations. It may be suggested that, while performing studies for the evaluation of mean portal vein diameter, it may be of great benefit to use similar methods which may lead to more accurate results and reduce measurement variations.

Although this study was performed by using a large number of subjects, it has some limitations too. The first one is that none of the subjects who were included in the study had any established or suspected liver disease, but still, in some of the subjects, liver function tests had not been performed. In addition, although the upper limit of normal mean portal vein was evaluated, this study did not include the patients with liver diseases and thus it was not possible to determine a predictive value differentiating the normal and pathological mean portal vein diameter values. Other studies conducted in the future, may prove useful to determine such a value.

As a conclusion, in this study it was found that the mean portal vein diameter with the value measured as 15.05 mm on computed tomography, was significantly higher than the generally accepted upper limit value of 13 mm (p <0.0001) and the mean diameter of the main portal vein measured by contrast-enhanced tomography was 0.26 mm wider than that of the main portal vein in non-contrast imaging.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gaziantep University School of Medicine (2018/245).

Informed Consent: Due to the retrospective design of the study, informed consent was not taken.

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Conflict of Interest: Authors have no conflicts of interest to declare.

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Review

HLA-G with Benefits and Damages: A Review

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ABSTRACT

Human leucocyte antigen-G (HLA-G) is a non-classical HLA that researchers primarily focused on association with the complications of pregnancy. Recent studies about the immunomodulatory characteristics of HLA-G showed that this molecule is not only important in pregnancy but also have effects on transplantation, autoimmune diseases, cancer and infectious diseases. The restricted polymorphic nature and the disease association has also showed in various studies. In this review it was aimed to evaluate the benefits and damages of the HLA-G by considering both the expression levels and polymorphisms.

Keywords: Cancer, HLA-G, immune tolerance, pregnancy, transplantation

INTRODUCTION

Human leukocyte antigen-G gene is located on the short arm of the 6th chromosome 6p21.3 and is one of the non classical HLA Class I genes (1,2,3). It is characterized by lower polymorphism with regard to the classical class I genes (4,5). The gene is consisted of eight exons and seven introns and co-dominantly expressed (2). Exon 1 encodes signal peptide, while exon 2, 3, 4 encode extracellular domain, alfa 1, alfa 2 and alfa 3, respectively. Exon 5 and 6 are encoded cytoplasmic and transmembrane domains. Exon 7 does not exist in mature mRNA because there is a stop codon in exon 6. Otherwise, Exon 8 is not translated into mRNA (3). HLA-G gene expression is regulated by miRNA's, nucleotide variations and epigenetic mechanisms (6). Environmental factors as growth factors, anti-enflamatuars, hypoxy, progesteron, interleukin 10, and interpherons can also effect HLA-G expression levels. Moreover, immunosuppressant agents can induce HLA-G expression (6).

There are 51 HLA-G alleles and 16 different variants (5). Therefore HLA-G has a highly limited peptide repertoire (7). These alleles encode 16 transmembrane proteins and two abbreviated proteins (8).

HLA-G gene, which is located between HLA-A and F genes, expresses four membrane bounded (G1, G2, G3, G4) three soluble (G5, G6, G7) isoforms which are formed by mRNA splicing. G1 and G5 are the isoforms of the most accomplished (9). There is also a soluble form of HLA-G1 that is formed by proteolytic cleavage (10). HLA-G1 and G5 consist of a heavy chain, three alpha globular domain which binds to non-covalently to beta-2 microglobuline and a peptide. G2 and G6 doesn't have any α-2 globular domains. G3 and G7 doesn't have α-2 and α-3, while G4 doesn't have α-3 globular domains. G5, G6 and G7 isoforms have a stop codon in exon 4. Therefore they don't have any transmembrane and cytoplasmic domains (6).

3' untranslated region (3'UTR) of the HLA-G gene has a variety of regulator elements that effect the mRNA splicing and stability as poly-A signal and AU rich patterns while 5' upstream regulator region regulates transcription (3, 11). 3' UTR of HLA-G arranged as seven haplotypes (UTR-1 to UTR-7) that can be detected in most of the populations. UTR-1 is the mostly expressed haplotype (4). Three polymorphic regions in 3'UTR can affect the HLA-G expression by different mechanisms as influence of mRNA stability, sHLAG protein expression and binding of microRNAs (miRNAs) (12). One of them is the 14 bp polymorphism (rs371194629) is associated with the mRNA stability and alternative splicing. The HLA-G isoform can be relevant to decreased sHLA-G levels. Second one is the C/G SNP at +3142 (rs1063320) position that affects the miRNA binding afinity. Ultimately, the decrease of the mRNA degredation and HLA-G production can be detected. The third one is the G/A SNP at position +3187 (rs9380142) which is related to decrease mRNA stability (4, 12).

HLA-G interacts with killer immunoglobulin-like receptor-2DL4, immunoglobulin-like transcript (ILT) receptor-4, and ILT2 ex-

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Table 1. Observations of HLA–G expression and polymorphisms according to the clinical situations

Observation	Type of Clinical Situation/ disease	Author
Uterine sHLA–G were decreased in infertility	İnfertility	Zidi et al. (9)
No significant expression pettern of HLA–G between recurrent misscarriage and healthy pregnancy	Recurrent misscarriage	Bhalla et al. (20)
Lower sHLA–G levels were detected in maternal blood of preeclempsia than healthy pregnancy	Preeclempsia	Yie et al., Steinborn et al., Yie et al., (21,22,23)
Lower HLA–G expression was detected in spontaneous abortus	Spontaneous abortus	Rizzo et al.(24)
Higher sHLA–G concentrations detected in serum in second trimester and before delivery	Obese pregnant women	Beneventi et al. (25)
HLA–G was not responsible for spontaneous abortus	Spontaneous abortus	Garcia–Laez et al.(26)
HLA–G 14 genotype has no effect on preeclempsia	Preeclempsia	Bermingham et al. (27)
HLA–G expression protect the cancer cells from immune response	Cancer	Paul et al. (30)
HLA–G expression increase in colorectal cancer patients	Colorectal cancer	Guo et al. (32)
HLA–G expression varies between tumor types	Cancer	Seliger et al. (33)
HLA–G expresion in trasplant patients were associated with better graft acceptance	Transplantation	Lila et al. (35)
Increased levels of HLA–G were reduced the risk of rejection in heart and comibend liver–kidney transplantation.	Liver–kidney transplantation Heart transplantation	Carosella et al. (36)
Decreased levels of HLA–G were increased the risk of rejection after kidney transplantation.	Kidney transplantation	Polankova et al. (37)
Increased sHLA–G concentrations were detected in rheumatoid disorder	Rheumatoid arthritis	Beneventi et al. (39)
14bp HLA–G allele frequency was increased in patients with Behcet's disease	Behcet's disease	Sakly et al.(42)
HLA–G 3741–3754ins14 variant is associated with increased risk for Behcet's disease.	Behcet's disease	Park et al. (40)
HLA–G010101 reduce the risk of Behcet's disease	Behcet's disease	Park et al. (41)
HLA–G 14 bp ins might increased the lupus erythematosus risk	Lupus erythematosus	Zhang et al. (43)
sHLA–G levels inversely correlated with ankylosing spondylitis	Ankylosing spondylitis	Zhang et al. (44)
HLA–G +3142 C/G polymorphism could have a role in susceptibility to coronary arter disease	Coronary arter disease	Gonen– Gross and Mandelboim. (29)
No associateion was observed betseen sHLA–G levels and schizophrenia	Schizophrenia	Rajasekaran et al. (45)
HLA–G can be associated with multiple sclerosis	Multiple sclerosis	Fredj et al. (46)
Increased levels of sHLA–G can cause to develop celiac disease	Celiac	Torres et al. (47)
Patients that have 14bp in/del polymorphism can have susceptibility to celiac disease	Celiac	Catamo et al. (49)

pressed on B lymphocytes, T lymphocytes, NK cells and antigen presenting cells (13, 14). ILT4 is a receptor for HLA-G free heavy chains while ILT2 recognizes b2-microglobulin (14).

HLA-G can inhibit immune response via different ways by interacting with these specific receptors (15). HLA-G restains T cells alloproliferation, cytotoxicity of CD8 (+) T and natural killer (NK) cells (9). Furthermore, HLA-G can bind to dendritic cells which results in inhibition of maturation, migration, trafficking antigen presentation and their association with T and NK cells (16). It also regulates the cytokine production of T helper-1 to T helper-2 (10). Soluble HLA-G molecules have the similar effects as membrane-bound ones (16). During pregnancy, HLA-G molecules expressed by the trophoblast cells were interacted with KIR on maternal NK cells. By

this way, immune tolerance develops and maternal immune system cannot be able to destroy the fetal tissues (17).

HLA-G antigen is known as an immunomodulatory molecule, and initially identified in cytotrophoblast cells of placenta (18). After then, the expression was observed on cornea, thymus, mesenchymal stem cells, pancreatic islets, and pathological conditions as cancers, viral infections, inflammatory and autoimmune diseases, and transplantation (5).

In this review we focused on the effect of HLA-G expression, polymorphism and serum levels on pregnancy, cancer, autoimmune disorders and transplantation.

CLINICAL AND RESEARCH CONSEQUENCES

Association of HLA-G and pregnancy

In various studies it was shown that HLA-G has an important effect on complications of pregnancy, as recurrent miscarriage, pre-eclempsia and spontaneous abortus. It was observed that expression of HLA-G isoforms decreased in pre-eclempsia and recurrent spontaneous abortus. It was also indicated that uterine sHLA-G levels were decreased in unknown infertility (9). Bhalla et al defined recurrent miscarriage (RM) as three or more pregnancy losses before 20 weeks. There was no significant difference in the expression patterns of HLA-G in women with RM compared with healthy pregnant women (20). Yie et al reported that at the end of last trimester lower concentrations of sHLA-G levels in maternal blood of pre-eclempsia than control pregnancies (21). This is supported by the studies in which sHLA-G levels were measured at second and first tirmester showed the same results (22, 23). Moreover, it was reported that spontaneous abortions, also showed lower levels of sHLA-G compared successful pregnancies (24). In another study that was performed among obese pregnant women, sHLA-G concentration in serum increased in the second trimester and before delivery, while in healthy controls it was decreased between the second trimester and the delivery (25).

García-Láez et al found HLA-G was not responsible for spontaneous abotions in the first trimester (26). On the other hand, several studies have observed lower levels of sHLA-G in pregnancies that have preeclempsia in the second and third trimester (12). The HLA-G 14 bp insertion polymorphism and serum HLA-G levels are also studied in women with pre-eclempsia (27). Berminham et al. established no influence of HLA-G 14 bp genotype on pre-eclempsia, while positive and negative associations to PE were reported (27). However, HLA-G 14 bp IN/DEL polymorphism in 30 UTR did not show any influence (28).

During pregnancy it was suggested that HLA-G molecule play a key role in preventing the rejection of the trophoblast cells by NK cells (29). Therefore HLA-G expression in pregnancy is one of the most important mechanisms for the maternal tolerance to the fetal cells.

Cancer and HLA-G

As an immune response regulator molecule, although HLA-G expression is beneficial in pregnancies, for cancer patients the expression levels in the tumor cells known to be harmful for the host. Paul et al. primarily described that HLA-G was expressed in the solid tumor and expression can protect the cancer cells from NK cytolysis for the first time (30). Since then, expression levels of HLA-G has been observed by immunohistochemical, western blot and qPCR in various solid tumors as breast, renal cell carcinoma, hepatocellular carcinoma, neuroblastoma, ovarian, lung, gastric and colorectal cancer, cervical carcinoma, retinoblastoma and malignant situations as B cell chronic lymphocytic leukemia, acute myeloid leukemia. However, there are controversial findings with regard to HLA-G prognostic values and expression levels (31).

HLA-G expression can be detected on exosomes that were derived from tumors in hematological and cell surface malignancies in addition to cell surface and secreted form. Recent studies showed that HLA-G could upregulated the expression of the tumor promoting factor. It was showed that HLA-G expression was strongly correlated to tumor metastasis, worse prognosis, and disease stage in tumor patients (19).

Guo et al investigated HLA-G and E levels of colorectal cancer patients and and detected increased levels by immunohistochemical methods (32). Otherwise, HLA-G expression significantly varies between tumor types. This could be due to the distinct antibodies used for the immunohistochemical methods (33).

Transplantation and HLA-G

The immunomodulatory characteristics of HLA-G promoted the investigators to determine the association between HLA-G levels, polymorphisms and rejection risk. Therefore HLA-G expression levels were studied after kidney, liver, heart, and liver-kidney transplantation (34). The results showed that HLA-G expression in transplant patients were associated with a better graft acceptance (35). Increased levels of HLA-G were associated with reduced acute and chronic rejection in patients that have heart and combined liver-kidney transplantation (36). Polakova et al detected the decreased HLA-G expression levels in serum and biopsy samples of the patients who had rejection attack after kidney transplantation. They also noticed that while expression levels were lower in the first week of transplantation, over time it increased (37).

Autoimmune diseases and HLA-G

The inhibition of the autoreactive cells by HLA-G expression can be advantegous for the patients with autoimmune disorders. In some studies this protective effect is indicated (38). Beneventi et al. detected higher s-HLA-G concentrations in pregnant women with autoimmune romatoid disorders than healthy pregnants (39). The association of the HLA-G polymorphism with Behcet's Disease was analyzed in different studies (40, 41). According to Park et al, HLA-G 3741_3754ins14 variant associated with imcreased risk for BD (40). On the other hand it was shown that HLA-G*010101 can be reduced the risk for BD (41). Sakly et al. showed that -14bp HLA-G allele frequency was increased in patients with BD (42).

Zhang et al. worked with the patients with Systemic lupus erythematosus (SLE) and observed differences between SLE and healthy controls in the allelic and genotypic frequencies of the

HLA-G 14bp ins/del polymorphism. They noticed that HLA-G 14 bp insertion allele can increased the SLE risk (43). In another study a significant association between Ancylosan Spondilit (AS) sacroiliitis stages and HLA-G expressions was reported (44). Zhang et al showed that while plasma sHLA-G levels were inversely correlated with AS, HLA-G expression on the cell surface can increase or decrease according to the stages of AS (44).

Association with other diseases

The associations between HLA-G levels and/or polymorphisms and several diseases have been worked by different groups. Zidi et al reported that the HLA-G +3142C/G polymorphism could be a genetic marker of susceptibility to coronary artery disease for the first time (29). Rajasekaran et al observed no significant association between sHLA-G levels and schizophrenia (45). It was reported that HLA-G allele can play a role in the development of multiple sclerosis. (46). Torres et al. demonstrated an association between Celiac Disease (CD) and soluble HLA-G (sHLA-G) expression. Increased levels of sHLA-G can be caused the succeptibility to develop CD. It was hypothesized by the authors that the increased expression of sHLA-G could play a role in the mechanism of gluten tolerance in CD in CD patients (47). Fabris et al have also shown the association between CD and HLA-G polymorphism (48). Catamo et al reported the 14 bp del/ins polymorphism in CD susceptibility (49).

The viral infections increased HLA-G expressionof the virus infected cells can also be a mechanism to escape from the host immune system. CD8+ T cells and monocytes were upregulated the expression of HLA-G in HIV-infected patients (50).

CONCLUSION

HLA-G expression is beneficial in transplantation, autoimmune diseases and especially pregnancy, in which it turns down the immune reaction against allograft, self components or the fetus. Otherwise in cancer and viral infections, it becomes having deleterious effect for the host cell. Consequently in future HLA-G based therapies can be developed for the pregnancy complications, transplantation and autoimmune diseases, while expression of it should be blocked in patients with cancer or viral infections. There are still some incompatible research results between working groups which tries to investigate the association between HLA-G and diseases. To elucidate this association, it is needed to perform multi- center studies including large study populations.

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Review

Novel Methods for Diagnosis Of Blood–Borne Protozoa

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ABSTRACT

The majority of parasitic infections are generally described in tropical and subtropical climate regions but affect developed countries due to the increase in migration and international travel. They may cause growth and development retardation in children, and labour and power loss in adults. As a result, rapid and reliable diagnosis forms the priority step for early treatment. In recent years, new and rapid diagnostic methods for diagnosis of blood parasites have been developed. Among these methods, serologic testing, rapid antigen tests, new nucleic acid amplification tests and proteomic methods come to the fore. In this review, methods still used for diagnosis of blood parasites are mentioned in brief and newly developed or developing methods are discussed.

Keywords: Blood-borne protozoa, diagnosis, methods

INTRODUCTION

Parasites causing diseases in humans are divided into two groups as protozoan (single-celled parasites) and metazoan (multi-celled parasites). Among blood-borne protozoan infections are the sporozoans of *Toxoplasma, Plasmodium and Babesia* and the flagellates of *Leishmania* and *Trypanosoma* (1, 2). Though the majority of parasitic infections are described in tropical and subtropical climate regions, developed countries are affected due to migration and international travel (1). Even those who have not travelled to endemic regions may be faced with blood-borne protozoan infections due to blood transfusions and organ transplants (3). Rapid diagnosis of parasitic infections is very important to determine appropriate treatment and prevent death. New techniques and tests used for diagnosis should be simple and rapid, prevent bias due to the user while interpreting test results. They also should have higher specificity and sensitivity (1). In our article, the basic methods used for laboratory diagnosis of blood-borne infections caused by protozoan pathogens are evaluated (Table 1) and new studies in the field of diagnosis are investigated.

CLINICAL AND RESEARCH CONSEQUENCES

Malaria

Plasmodium genus infects a wide range of birds, mammals, reptiles, and amphibians on earth using blood-feeding dipteran insects. Although there are at least 200 named *Plasmodium* species, only five infect mankind: *P. falciparum, P. malariae, P. vivax, P. knowlesi and P. ovale* (4).

Classical microscopy

Microscopic examination of thin and thick blood smears stained with giemsa dye is the classical method for diagnosis of malaria. Accurate interpretation varies according to the availability of trained and experienced laboratory technicians, quality of reagents and light microscopes. The thick film contains 2 drops of blood that have been lysed on the slide by addition into a hypotonic solution. This releases intracellular parasites and helps examination of up to 30 layers of blood. Thick blood film is more sensitive (20 times) than the thin film, with a reported detection threshold of 10 to 50 parasites/µl of blood, or approximately 0.0002 to 0.001% parasitemia. Because of this high sensitivity, a thick film is ideal for screening and parasite detection. Under field conditions, the estimated sensitivity may be lower (100 to 500 parasites/µl of blood) (4). Microscopic examination of thick and thin blood films have some extra benefits for diagnosis of malaria. Microbiologists can show the presence of species and parasitemia by preparing smears in short time (less than an hour). It is also possible to distinguish asexual forms and show mixed species causing infection by this method. The microscopic examination can also give information about the morphology and quantity of blood cells (5).

Other Microscopy Methods

Other less common microscopic methods are used for the identification of malaria parasites in the whole blood, including staining methods for nucleic acid and hemozoin. Acridine orange (AO), a DNA-binding fluorescent dye, excites at 490 nm and produces a yellow or apple-green fluorescence. This method

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Table 1. Diagnosis of blood–borne protozoan infections (1)

Pathogen	Microscopy	Serology	Molecular	Proteomic
Plasmodium spp	Detection from blood smear	RDTs	LAMP, LAMP card test, realtime PCR	LDMS
Babesia microti	Examination of blood smears to detect parasite in the patients' RBCs	IIF, ELISA, immunoblot	PCR, realtime PCR	
Leishmania spp	Detection of parasites in aspirates from spleen, bone marrow, or lymph nodes	ELISA, ICT strip test	PCR, NASBA, oligochromatography	
Trypanosoma brucei	Detection of parasite (trypomastigote) in the blood or CSF	CATT, micro–CATT and LATEX (T. b. gambiense)	PCR, LAMP, realtime PCR	SELDI–TOF
Trypanosoma cruzi	Detection of parasite (trypomastigote) in blood smears	IIF, IHA, ELISA, immunoblot, radioimmunoprecipitaton, mix–ELISA, TESA–ELISA	PCR	SELDI, MALDI MS–MS
Toxoplasma gondii	Detection of parasites from blood, CSF or stained tissue	Sabin–Feldman dye test, IIF, hemagglutination, capture ELISA, ISAGA, avidity ELISA, γinterferon ELISA	PCR	

ELISA, enzyme–linked immunosorbent assay; ICT, immunochromatographic; PCR, polymerase chain reaction; NASBA, nucleic acid sequence–based amplification; CSF, cerebral spinal fluid; IIF, indirect immunofluorescent; ISAGA, immunosorbent agglutination assay; CATT, card agglutination test for trypanosomiasis; LAMP, loop–mediated isothermal amplification; LATEX; rapid latex agglutination test; SELDI–TOF, surface–enhanced laser desorption ionization time–of–flight mass spectrometry; MALDI–TOF, matrix–assisted laser desorption ionization time–of–flight mass spectrometry; IHA, indirect hemagglutination; TESA, trypomastigote excreted–secreted antigens; MS–MS, tandem mass spectrometry; RDT, rapid diagnostic test; LDMS, laser desorption mass spectrometry; RBC, red blood cell

requires a fluorescence microscope or light microscope with an adaptor. Some studies found the AO method has similar sensitivity and specificity to traditional Giemsa-stained thick films. It can reliably detect <100 parasites/µl or 0.002% parasitemia and allows for more rapid screening than with traditional Giemsa method. Malaria hemozoin pigment may be detected by dark field microscopy or in histological tissue sections. Another quantitative buffy coat (QBC) method requires a fluorescent microscope and has high sensitivity for *P. falciparum* (4).

Serology and Rapid Diagnostic Tests

P. falciparum Histidine-Rich Protein 2 (PfHRP2), which exhibits polymorphism, is widely used as a diagnostic marker. Verma et al. (6) developed monoclonal antibodies (mAbs; b10c1 and Aa3c10) against 105th amino acid at the C terminal of histidine-rich protein 2 antigens of *P. falciparum* and used it in studies. The researchers determined the sensitivity and specificity of the monoclonal antibodies as 95% and 96%. This data strongly suggests that the anti-C-terminalPfHRP2 mAbs b10c1 and Aa3c10 have merits for improving the existing malarial diagnostics. Rapid diagnostic tests for malaria included serology-based tests for use especially in field studies (7). Rapid Diagnostic Tests (RDT) started to gain importance for detecting *Plasmodium* species. These tests are used to investigate malarial antigens and generally are in the forms of dipsticks or immune chromatographic cards (5). There are some target antigens for these tests. Histidine-rich protein-2 is produced only by *P. falciparum*. Plasmodial lactate dehydrogenase may be used to investigate *P. falciparum* or the genus. Aldolase enzyme may detect *Plasmodium* genus. Various RDTs can singly detect *P. falciparum*, *Plasmodium vivax* or pan-malaria species (5). In order to be placed on the World Health Organization procurement paper, *P. falciparum* test panel must get at least 75% detection score at 200 parasites/µl, for *P. vivax* panel it should be 75% or more at 200 parasites/µl, additionally false positivity should be less than <5% (8). Rapid tests are fast, practical, need minimum samples, but they do not seem to get the place of microscopy due to some disadvantages that they do not determine parasitemia, cannot differentiate sexual and asexual stages, and cannot make specific diagnosis for the pathogens of *P. ovale, P. knowlesi,* or *P. malariae* (5). Additionally, they cannot efficiently detect infections due to *P. falciparum* in South America, because of the lack of common histidine-rich proteins in this species (1). Advantages and disadvantages of malaria rapid diagnostic tests are shown in Table 2.

Molecular methods

Polymerase chain reaction (PCR) is commonly used for nucleic acid amplification and detection of *Plasmodium* parasite DNA, with the 18S small subunit rRNA gene. Most of the PCR are developed in research laboratories. There are some commercial PCR tests, but none of these were confirmed by Food and Drug Administration (FDA) (4). Poon et al. (9) developed a loop-mediated isothermal amplification (LAMP) test identifying the *P. falciparum* 18S rRNA gene *in vivo*. When they compared this method with PCR, they found the sensitivity was 95% with a specificity of 99%. In recent times, the LAMP has been further simplified as card test.

Table 2. Advantages and disadvantages of malaria rapid diagnostic test (1, 4, 5)

Advantages	Disadvantages
Rapid and easy to use	Do not measure parasitemia
Subjective result	Do not distinguish sexual–asexual stages
Supports microscopy	No species specific diagnosis for P.ovale, P.malariae, P. knowlesi
Requires minimal patient samples	More expensive than blood smear
Appropriate for field use	Inefficient detection for P. falciparum in South America

Yamamura et al. (10) used LAMP in combination with DNA filtering paper and analysis of the melting curve to diagnose *P. falciparum* and reported sensitivity was 97.8% and specificity was 85.7% when compared with microscopic method. Lee et al. (11) used a multiplex PCR method able to identify *P. knowlesi.*

Other diagnostic laboratory methods

Rapid diagnosis of malaria is very important for control of the infection. Investigation of hemozoin pigment of plasmodium via laser desorption mass spectrometry (LDMS) had been evaluated as a sensitive (<10 parasites/μL) method to detect *P. falciparum* species cultured in human blood. In mice, hemozoin pigment had been detected via LDMS in 0.3 μL of blood within two days of infection independently of the inoculating dose of 10^6, 10^4, or 10^2 parasite-infected erythrocytes. Investigators suggested that LDMS test for hemozoin may become a faster screening test compared to light microscopy for low-level parasitemia <0.1% (12, 1). Additionally, LDMS test was shown to become a faster and more sensitive alternate test than microscopy in a pregnant woman (13, 1). Recently, a device was designed for rapid detection of *P. falciparum* in malaria patients using a non-invasive way. The principal of the test was based on detecting the vapour bubbles around the hemozoin via transdermal optical excitation and acoustic detection. This instrument was suggested as cheap and practical diagnostic tool that can be useful for clinicians and researchers in the field. But, the test still needs to be developed and evaluated by using multiple cases (14).

Babesiosis

Babesia genus infects wild and domestic animals worldwide via primarily ixodid tick vectors. There are more than 100 named *Babesia* species but only several are known to regularly infect humans. *Babesia microti* is causes the majority of infections in human in the United States. *B. duncani* infections have also been reported here. Most cases of this infection are due to the bite of an infected ixodid tick (4).

Microscopy

Just like malaria diagnosis, the traditional diagnostic method for babesiosis is classical microscopy using thick and thin blood films stained by Giemsa dye. *Babesia spp*. may present a diagnostic challenge on blood films since there are many morphologic similarities shared with *Plasmodium spp*. (specifically *P. falciparum*) (4). Generally, *Babesia* parasites demonstrate greater pleomorphism in size and shape compared to *P. falciparum* parasites. Ovoid, elliptical, pear, racket, and spindle shapes may commonly be seen. It is not possible to differentiate the various human *Babesia spp*. by their morphological appearance. Molecular methods are required for identification of the species (4).

Isolation procedures

A biotest was performed, via inoculation of patient's blood into the peritoneum of laboratory rodents to confirm the disease. However, it is difficult to employ such labor-intensive methods in routine testing and it requires a long waiting time to receive the result (2–4 weeks) (15, 16).

Serologic methods

In the diagnosis of babesiosis, commercial serological tests are used, e.g. immunofluorescence assay for detecting immunoglobulin M (IgM) or immunoglobulin G (IgG) antibodies versus *B. microti* (15, 16). Antibodies against *B. microti* antigens typically appear 2 weeks after the onset of illness. They could be detectable for several years after infection. The indirect immune fluorescent assay (IFA) is the recommended diagnostic method. It detects serum antibodies against *B. microti* with a relatively high sensitivity (88 to 96%) and specificity (90 to 100%) (4). Though there are enzyme-linked immunosorbent assays (ELISA) and immunoblotting methods, there is still no standardization for these tests and they require confirmation with IFA (1).

Molecular methods

Currently, PCR is the reference method for the diagnosis of babesiosis (16). PCR is recommended if the species of pathogen cannot be identified based on the blood smear or if the diagnosis is unclear and medical history and clinical symptoms indicate babesiosis infection (15, 16). The sensitivity of PCR for 18S rRNA gene was assessed at between 5–10 pathogens/μl of blood which corresponds to 0.0001% parasitemia level (16). Rozej-Bielicka et al. (17) developed a multiplex PCR method to identify pathogen species including *B. divergens, B. microti, B. venatorum* and *B. canis* in a variety of biologic samples. The researchers state the method was practical and cheap for use in screening and for diagnostic purposes. Currently, there are no FDA-approved *Babesia* PCR assays (4).

Leishmaniasis

Leishmania spp. are protozoal members of the family Trypanosomatidae. The infection due to this pathogen is called leishmaniasis and is a zoonosis of obligate intracellular parasites transmitted to humans by bites from infected female sand flies. Depending on the species, *Leishmania spp*. infection can manifest with different forms such as cutaneous, diffuse cutaneous, mucocutaneous, or visceral disease (18). Various microscopic and cultural methods were developed to detect this parasite (18-20)

Microscopy

The gold standard method for diagnosis of visceral leishmaniasis (VL) is classical microscopy. The specificity of the method is high; however, sensitivity is variable (20). Amastigotes may be

recognized by size, shape, staining properties, and the presence of a kinetoplast. After Giemsa staining, the cytoplasm will appear bluish, and the kinetoplast and the nucleus will appear red-purple. Since amastigotes cannot be stained with mucicarmine, periodic acid-Schiff, or silver stain, we can differentiate them from intracellular fungi by using these dyes (18).

Culture

For culturing, samples must be collected aseptically. Tissues should be minced before culturing. Schneider's Drosophila medium with 30% fetal bovine serum and Novy, MacNeal, and Nicolle's medium (NNN) can be used as culture media. Culture media, incubated at 25 °C, could be checked two times a week for the first 2 weeks and once a week thereafter for up to 1 month before the culture is declared negative. Promastigote stages of the pathogen can be detected microscopically in wet mounts (18). In 2004, a microcapillary diagnostic test based on culturing method was developed for cutaneous leishmaniasis (CL). Higher sensitivities and shorter periods for promastigote emergence were reported in this method (19).

Animal inoculation

For diagnosis of leishmaniasis, animals like hamsters can be inoculated with the patient material. For cutaneous and mucocutaneous leishmaniasis animals should be inoculated intranasally and for VL, they should be inoculated intraperitoneal. Positive identification in the animal may take 2-3 months (18).

Serology

The most hopeful antigen for serologic diagnosis of visceral leishmaniasis are antigens related to kinesin. An immunochromatographic strip test developed using rK39 antigen can be used for mass screening in endemic regions (21). Magalhães et al. (22) tested three antigens mixtures (poly-histidine tagged polypeptides) and found it was useful for canine or human visceral leishmaniasis, among 13 identified through different screenings. This investigation gave similar results with high sensitivity for both canine (88%) and human (84%). In a recent study, enolase enzyme of *L. braziliensis* was cloned and rEnolase recombinant protein was tested for serodiagnosis of canine and human VL. As a result, ELISA test with rEnolase indicated 100% diagnostic sensitivity and 98.57% diagnostic specificity for canine VL, and 100% and 97.87%, respectively, for VL in human. It was reported that searching the antibodies against rEnolase improved the serodiagnosis of VL (23). Coelho et al. (24) evaluated the diagnostic properties of cytochrome c oxidase and IgE dependent histamine-releasing factor proteins in canine VL and human tegumentary leishmaniasis. ELISA tests using these recombinant proteins showed 100% sensitivity and specificity for serodiagnosis of both infection forms. These proteins showed a better diagnostic performance than the *Leishmania* antigen extraction or recombinant A2 protein (24).

Molecular methods

PCR is the most important molecular diagnostic method for *leishmania* infections because of its high sensitivity and reliability. Some PCR protocols use different targets such as ribosomal RNA, DNA of kinetoplast, mini-exon RNA or internal transcribed spacer (ITS) (1). In addition, assessment of antimicrobial drug treatment and determination of clinical outcomes can be carried out through the use of nucleic acid sequence–based amplification (NASBA) by amplifying the RNA sequences. In combination with oligochromatography, NASBA may be used for monitoring the progression from active disease to cure (25). Niazi et al. (26) developed a nano diagnostic method using a NASBA method and gold nanorods for colourimetric measurement targeting 18 S rRNA of *Leishmania* and reported the sensitivity was 100% with a specificity of 80%. In a study in Iran, ITS-rDNA, Hsp70, and Cyt b genes were used for accurate identification of *Leishmania spp* and investigated in clinical samples from three important regions where CL is common. By using the combination of three genes, 231 *Leishmania* parasites were identified correctly among 360 clinical samples and this method was found to be more sensitive than routine laboratory methods that can only detect 203 *Leishmania* parasites (27). Sagi et al. (28) developed a practical swabbing test, combined with highly sensitive multiplex PCR for detection of *Leishmania* infections. They found that this combination was very practical and more sensitive than classical microscopy (28). Multilocus sequence typing (MLST) and Multilocus enzyme electrophoresis (MLEE) are used to identify *Leishmania* species and strains, but this depends on having culturable isolates, and in some cases, these methods were not discriminative enough (18).

Protein analysis methods

In recent years, proteomic methods started to gain importance in the diagnosis of parasitosis. Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) spectrometric method was used to detect *Leishmania* pathogens isolated from cultures (29). The researchers reported they created a free web-based application for *Leishmania* species and set up a data library containing fingerprints of the pathogens' spectra. Researchers also identified differentially expressed proteins in the inflammatory region of CL, revealed increased expression of caspase-9. Immunological analyses validated the involvement of caspase-3, caspase-9, and granzyme B in tissue damage in CL cases (30). Duerte et al. (31) investigated some antigenic proteins for validation of the results of CL immunoscreening. They investigated the coding regions of some antigens such as enolase, tryparedoxin peroxidase, eukaryotic initiation factor 5a, and beta-tubulin. After being cloned in a vector, these proteins serodiagnostic performances were evaluated for CL. These proteins had sensitivity and specificity levels ranging from 82.5 to 100%. The study suggested the use of these antigenic proteins for diagnosis of CL (31).

African trypanosomiasis

African trypanosomiasis is generally seen in the tsetse fly belt of Mid-Africa. The Gambian form of sleeping sickness, noted for its chronicity and responsible for 99% of the sleeping sickness cases, is caused by *T. brucei gambiense*. Rhodesian (East African) form, noted for its acute morbidity and mortality within months of infection, is caused by *T. brucei rhodesiense* (18).

Microscopy

In addition to staining thin and thick blood smears, determining the buffy coat is recommended to detect the parasites. Parasites

can be detected on thick blood smears when numbers are greater than 2,000/ml, by determining the hematocrit concentration in a capillary tube or by quantifying buffy coats when numbers are greater than 100/ml, and by anion-exchange chromatography when numbers are greater than 4/ml (32). In suspected and confirmed cases of trypanosomiasis, a lumbar puncture is mandatory to rule out central nervous system involvement. Cerebrospinal fluid (CSF) examination must be conducted by using centrifuged sediments (33).

Culture and Animal Inoculation

Rats and guinea pigs have also been used to detect trypanosomiasis. *T. brucei rhodesiense* is more adaptable to cultivation (Tobie's medium) and animal infection than *T. brucei gambiense;* however, cultivation methods are not practical for most clinical laboratories (18).

Serologic methods

Serologic techniques including IFA, ELISA, indirect hemagglutination assay, card agglutination trypanosomiasis test (CATT), and LATEX/*T. b. gambiense* has been used for epidemiologic screening. However, these methods have not been approved by the FDA. Serologic tests are normally used for screening. For a definitive diagnosis, microscopic observation of trypomastigotes is needed (18). A card agglutination test (CATT/*Trypanosoma brucei gambiense*) developed in 1978 for West African Trypanosomiasis diagnosis is a cheap, rapid and high sensitivity test; however, it may give high false positives for infections accompanied by malaria (1). Similarly, micro-CATT and LATEX/*T. b. gambiense* tests are used especially in endemic regions; however, they need to be confirmed by microscopy (1). The CATT and LATEX/*T. b. gambiense* have good negative predictive values. Markedly elevated serum and CSF IgM concentrations have diagnostic value (18).

Molecular methods

For diagnosis of African Trypanosomiasis infections, the *T. b. rhodesiense* serum resistance-associated (*SRA*) gene is used with PCR and LAMP techniques in CSF samples from patients (34, 35). Becker et al. (34) completed real-time PCR with primers synthesized to target the 177 bp repeated satellite DNA of the parasite. The researchers reported the method was rapid and sensitive for use in routine laboratories. Diagnosis of African Trypanosomiasis infections was researched with the *SRA* gene used with PCR and LAMP techniques in CSF samples from patients (34, 35).

Protein analysis methods

Recently, researchers reported the discovery of serum proteomic signature for diagnosis of human African trypanosomiasis by using surface-enhanced laser desorption-ionization time-of-flight (SELDI-TOF) mass spectrometry and data-mining algorithms. The new method, coupled with biochemical characterization of the proteins that contribute to the signature, provides stronger and novel tools to create improved diagnostic tests (36).

American trypanosomiasis

Chagas' disease also called American trypanosomiasis which is a zoonotic infection is caused by *Trypanosoma cruzi*. Triatomine insect infected with protozoa from other contacts with animals transmits the trypanosomes when the triatomine deposits its faeces on the skin of the host and then bites (18).

Microscopy

Trypomastigotes can be detected by using wet mounts of blood, examining blood smears or the concentrated buffy coat. Giemsa stain is used for both amastigote and trypomastigote stages. *L. donovani* and *T. cruzi* infections can be differentiated by other methods such as PCR, immunoassay, culture, serologic tests and animal inoculation (18).

Culture and Animal Inoculation

In laboratories, aspirates, blood, and tissues can be cultured. Generally, the NNN medium is chosen. Cultures, incubated at 25 °C, should be examined for epimastigote forms at least two times a week during the first 2 weeks and once per week thereafter for up to 1 month before considered as negative. In advanced laboratories, rats or mice may be inoculated and their blood can be investigated for trypomastigotes (18).

Xenodiagnosis

In this method, trypanosome-free reduvid bugs are allowed to feed on individuals suspected of having Chagas' disease. Faeces, hemolymph, hindgut, and salivary glands can be examined microscopically for flagellated forms over a period of 3 months, or PCR methods can be used to detect infected bugs and provide a rapid diagnosis (37). Xenodiagnosis is positive in less than 50% of seropositive patients (18).

Serologic methods

Blood and saliva are used for the diagnosis of Chagas' disease. Complement fixation (Guerreiro-Machado test), chemiluminescence, IFA, indirect hemagglutination, and ELISA are employed for serological testing. Most of these tests use an epimastigote antigen, and cross-reactions have been reported for patients infected with *T. rangeli, Leishmania spp*., *Toxoplasma gondii*, and hepatitis (18). Secretory antigens of *T. cruzi* have potential to be used in the serologic diagnosis of Chagas disease. Umezawa et al. (38) developed recombinant antigens including B13, 1F8 and H49 antigens to create a *T. cruzi* mix ELISA kit. The sensitivity and specificity of the kit were determined as 99.7% and 98.6%, respectively. Sánchez-Camargo et al. (39) evaluated 11 different RDTs for detecting *T. cruzi* antibodies in Serum Banks. These tests relied on different testing principles such as particle agglutination, immunochromatography, immunofiltration, or immunoblot. They found that 8 out of 11 tests were useful to detect infections (39).

Molecular methods

PCR test was used to detect as few as one trypomastigote in 20 ml of blood and was found useful in treatment follow-up. However, it is not routinely available in the field. A real-time PCR using multiple gene targets has been advocated to improve on detection of positive patients. More target genes are needed due to polymorphism (18).

Protein analysis methods

New data suggest that both MS platform-dependent and platform-independent biomarker-based tests may be beneficial for

Table 3. Guide to general interpretation of Toxoplasma serology results obtained with IgG and IgM commercial assays (2)

IgG result	IgM result	Report/interpretation for humans (except infants)
Negative	Negative	No serological evidence of infection with Toxoplasma
Negative	Equivocal	Possible early acute infection or false-positive IgM reaction. Obtain a new specimen remain the same, the patient is probably not infected with Toxoplasma.
Negative	Positive	Possible acute infection or false-positive IgM result. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the IgM reaction is probably a false positive.
Equivocal	Negative	Indeterminate. Obtain a new specimen for testing or retest this specimen for IgG in a different assay.
Equivocal	Equivocal	Indeterminate. Obtain a new specimen for both IgG and IgM testing.
Equivocal	Positive	Possible acute infection with Toxoplasma. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same or if the IgG test becomes positive, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Negative	Infected with Toxoplasma for more than 1 year.
Positive	Equivocal	Infected with Toxoplasma for probably more than 1 year or false-positive IgM reaction. Obtain a new specimen for IgM testing. If results with the second specimen remain the same, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Positive	Possible recent (within the last 12 months) infection or false-positive IgM reaction. Send the specimen to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.

subjects with latent Chagas Disease. Rather than replacing antibody-based and PCR testing, it seems that mass spectrometry assays will help build more complementary information about the diagnosis for future (1).

Toxoplasmosis

Toxoplasma gondii protozoan parasite causes the infection toxoplasmosis and it is one of the most common parasitic infections in humans. This disease is most typically asymptomatic. However, in select clinical situations, it can cause severe disabilities. So, it is important to make an accurate and timely diagnosis (2).

Microscopy

Diagnosis by the microscopic examination of patient samples is very rare. Secretions, exudates, sterile fluids and tissues are potential samples for direct observations; however, they are generally not chosen. Giemsa staining in CSF (40) or heparinized fluid samples fixated in methanol may identify *T. gondii* tachyzoites, while the same sample may identify *T. gondii* cysts in tissue samples (2).

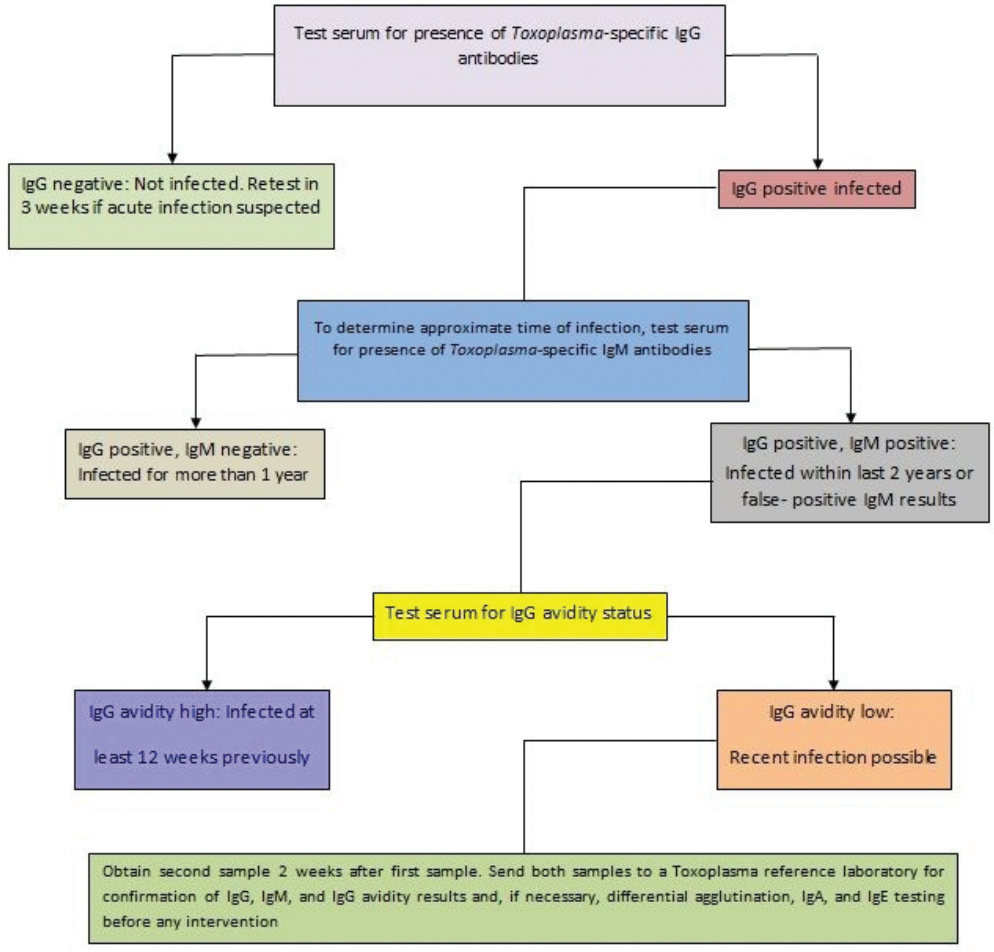
Isolation procedures

Toxoplasma gondii isolation can be performed by inoculating patient sample into either mice or cell culture. But the success of this method is limited. *T. gondii* grows in tissue culture cells. Cytopathic effects may be shown on direct examination after 24 to 96 hours. Isolation in the cell culture technique allows faster diagnosis than the inoculation of microorganism in mice (2).

Serologic tests

The Sabin-Feldman dye test has been accepted as gold standard method since it has high sensitivity and specificity (1). Several laboratories have gone on using this method, while most laboratories focus on novel methods including immunofluorescent antibody assays, hemagglutination tests, immunosorbent agglutination assays, and capture ELISAs (1). *Toxoplasma*-specific IgM antibodies have been used to differentiate between chronic and acute infections. The most important use regarding IgM test results is that a negative reaction essentially excludes recent infection. A guide for interpretation of *Toxoplasma* IgG and IgM serology results is presented in Table 3 (2). The *Toxoplasma* IgG avidity test is an important tool to discriminate between past and recently acquired infections. During acute infection, IgG antibodies bind antigen weakly or have low avidity, whereas, during chronic infection, antibodies bind antigen more strongly or with high avidity. So, the avidity test works on this principle. A high-avidity result indicates infection acquired more than 3 to 5 months before. But low-avidity result does not indicate a recently acquired infection since low-avidity antibodies may be detectable for a year post-infection (2). Testing for *Toxoplasma*-specific IgA antibodies should be performed in addition to IgM assays for newborns which are suspected to have a congenital infection. *Toxoplasma*-specific IgE antibodies may also contribute to the determination of acute infections, although reports of the utility of IgE antibody detection have been mixed (2). An algorithm for serological testing for immune status and acute acquired infection is shown in Figure 1. A new method developed for the postnatal diagnosis of toxoplasmosis is based on measuring interferon-gamma levels in full blood cells stimulated by *Toxoplasma* antigens with ELISA (41). The interferon gamma release test (IGRA) shows activation of lymphocytes after *T. gondii* antigen stimulation, and distinguishes infected individuals from those who are not infected. This test is practical and economical method to show the cell-mediated immunity against the pathogen (42). Begeman et al. (43) used the *Toxoplasma* ICT IgG-IgM point of care test for diagnosis of congenital toxoplasmosis (CT) and showed the new test was 100% sensitive and specific for identifi-

Figure 1. Algorithm for serodiagnosis of toxoplasmosis in the people older than 1 year of age



cation of *Toxoplasma* infections. Zacche-Tonini AC et al. (44) evaluated the conventional serology (Q-Preven™ and ELFAVIDAS™) and flow cytometric assays for early serodiagnosis of CT. In conclusion, they proposed a novel algorithm with high accuracy (97.1%). This includes screening with Q-Preven™IgM assay at the birth, followed by flow cytometric IgG avidity analysis and ELFAVIDAS™ IgM during the first month after birth. It was claimed that these assays had a high-performance for early serological diagnosis of CT. Baschirotto PT and co-workers (45) designed a novel test using liquid microarray method. They evaluated different antigens to detect IgGs against *T. gondii* and *rubella*. The performance of 6 out of 13 antigens was sufficient to be used in a multiplex PCR assay for diagnosis of the *T. gondii* infection. The test was reported to have 100% sensitivity and specificity for detection of *T. gondii* infection. The test seems to have a potential for prenatal infection screening of the pregnant women after some modifications (45).

Nucleic acid detection methods

Recently, different molecular methods including PCR, real-time PCR, nested PCR, and also LAMP were designed for diagnosis of toxoplasmosis. PCR test was found useful to detect pathogens in amniotic fluid, placental and cerebral tissues, and aqueous humor and vitreous fluid (46). PCR method with amplification of repeating B1 gene of *T. gondii*, 18S rRNA, P30, 529-bp repeat fragment or AF146527 element was used for molecular diagnosis. Nested PCR was used to increase specificity of DNA amplification and it was found useful to detect the pathogens which are found in low amounts in the specimens (46, 47). Berredjem et al. (48) used PCR studies of peripheral blood samples and amniotic fluid samples for early toxoplasmosis diagnosis in pregnant women. Samples with PCR amplification were divided into two, with nested PCR to increase *T. gondii* in the B1 region and PCR-ELISA using major surface antigen P30 gene primers. With regards to the PCR assay using peripheral blood and amniotic fluid (AF), both B1 and P30 primer sets performed equally well and therefore appear adequate for *Toxoplasma* identification. However, B1 gene proved valuable PCR for *T. gondii* detection better than P30 gene (48). Mousavi et al. (49) reported that the performance of the B1 gene was better than the RE gene for molecular diagnosis of toxoplasmosis. Real-time PCR seems to be a very sensitive molecular diagnostic test which can detect the DNA region even at low concentrations. In addition, these tests are fast, more sensitive and reproducible when compared to classical PCR. They

can also be used for monitoring the therapeutic response and prognosis of the infection (46). It is also possible to investigate parasite load by using this method. Varlet-Marie E et al. (50) evaluated a novel test, the Iam TOXO Q-LAMP (DiaSorin, Italy) assay, using a reference real-time PCR method (laboratory developed). This LAMP method was found to be less sensitive than real-time PCR at very low parasite load. However, both methods yielded identical results qualitatively.

CONCLUSION

Although novel and rapid diagnostic instruments are being investigated and even used by advanced microbiology units, the results often need to be confirmed by microscopy, which is accepted as gold standard (1). Commercially available rapid antigen and antibody detection kits are easy to use and suitable for mass screening. However, rapid tests are expensive, are not the gold standard and may give false negative results. Molecular tests including PCR, real-time PCR, NASBA, oligochromatography, and LAMP have improved during the past decade. PCR methods for all six blood parasites and LAMP technique for *Plasmodium spp* and *Trypanosoma brucei* can be used for diagnosis of parasitic infections. Recently, MALDI MS, LDMS and SELDI-TOF proteomic techniques that analyze the protein expression of the parasites have begun to give promising results and opened new horizons for the future of diagnosis of blood-borne parasites. However, new and larger studies with different species are still needed for the standardization and optimization of these novel techniques.

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Case Report

Rare Cause of Cerebellar Mutism in Childhood: Vertebral Artery Dissection

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ABSTRACT

Vertebral artery dissection (VAD), an extremely rare childhood disorder, constitutes 2%-3% of cerebrovascular diseases (CVDs). The annual incidence of CVD in childhood is estimated to be 3-8 per 100.000. Although it is generally observed after trauma, it could also be observed simultaneously with trauma. CVD is diagnosed by cranial magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA). However, the gold standard for CVD diagnosis is conventional angiography. A 12-years-old girl presenting with symptoms of headache and vomiting lasting for 5 days was hospitalized and followed up. In the neurological examination, muscle power in lower and upper extremes was 4/5 on the right and 2/5 on the left, while Babinski response was extensor on the left. Cranial MRA revealed a dissection in the right middle vertebral artery at the M1 segment, with vascular irregularity. Warfarin sodium was administered to the patient based on recommendation, and physical therapy was also started. Moderate recovery was observed in aphasia, and partial recovery was observed in left hemiplegia after 7 days of treatment. Cerebellar infarct and cerebellar mutism cases should be investigated for VAD. Early diagnosis and treatment are very pertinent for reduction of mortality and morbidity due to the disease. Angiography should be performed for diagnosis.

Keywords: Childhood, stroke, vertebral artery dissection

INTRODUCTION

The annual incidence of cerebrovascular disease (CVD) in childhood is estimated to be 3-8 per 100.000 (1). Vertebral artery dissection (VAD), an extremely rare childhood disorder, accounts for 2%-3% of CVDs (2). Since vertebral and basilar arteries are deeply located in the body, surgical intervention is often difficult, thereby posing a very high risk. Although CVD is generally observed after aneurysm, it can also occur spontaneously or due to connective tissue diseases such as Ehler-Danlos syndrome type 4 and Marfan syndrome (1, 2). VAD is generally associated with stroke due to deterioration of posterior circulation in the brain and also due to bulbar symptoms, mutism, dysarthria, vertigo, and focal findings (3). Diagnosis of VAD is determined by cranial magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA). However, the gold standard for diagnosis is conventional angiography (4). Intravascular thrombolysis, thrombectomy, and anticoagulants may be used as interventions for the treatment of VAD (5).

CASE PRESENTATION

A 12-year-old girl presenting with symptoms of headache and vomiting lasting for 5 days was hospitalized and followed-up. No anomaly was observed in the biochemical analysis and cranial computed brain tomography. After the inclusion of aphasia in the clinical symptoms, lumbar puncture revealed no cells in the cerebrospinal fluid. MRI was done in the follow-up, and the patient was directed to our clinic with suspicions of a mass in brain stem, and acute ischemia.

Cardiac apex beat was 98/min, arterial blood pressure was 104/68 mm/Hg, and fever temperature was 37^{0}C. Aphasia was observed and the patient responded appropriately to verbal signs and commands. In the neurological examination, muscle power in lower and upper extremities was 4/5 on the right and 2/5 on the left, and Babinski response was extensor on the left. The patient showed no signs of meningeal irritation while other examinations were normal. In the focal neurological examination, the patient showed a ischemic foci involving partial contrast in the cranial MRI, with bilateral diffusion limitation in thalamus and pons, which was more apparent on the right (Figure 1). Cranial MRA revealed a dissection in right middle vertebral artery at the M1 segment, with vascular irregularity (Figure 2). Enoxaparin sodium followed by warfarin sodium were administered. The patient had no history of trauma associated with the dissection. Thrombophilia panel was determined as MTHFR A1298C heterozygote and GPIIIa L33P heterozygote. Warfarin sodium was continued, while physical therapy was administered. Moderate recovery was determined in aphasia, and partial recovery was determined in left hemiplegia after 7 days of treatment. After 14 days of treatment, there was almost a total recovery in aphasia,

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and a moderate recovery in left hemiplegia. INR level was maintained between 2.5 and 3.5. The patient was discharged, with a continuation warfarin sodium treatment and regular physical therapy. After the discharge, muscle power was 5/5 on the right and 4/5 on the left after 45 days of treatment. She was walked unsupported with hemiplegic gait on the left side, with a complete recovery of her speech.

Written informed consent was obtained from the patient's family.

DISCUSSION

VAD can occur following a minor trauma or even spontaneously. It is an extremely rare disorder in childhood (1, 2). Although family history of bleeding diathesis has been detected in some cases, there is no proven pre-disposing factor for the disease (6). No trauma, syndromic findings, bleeding diathesis, and family history were observed in the present case.

Patients present different clinical symptoms based on the area affected by VAD. Patients generally present bulbar symptoms, dysarthria, vertigo, and focal symptoms (7). Although our patient had clinical symptoms of headache and vomiting, aphasia and focal symptoms were later included. Acquired neurologic childhood mutism may develop as a result of damage in different areas of the brain. Cerebellar mutism is rarely diagnosed in children. The most common reasons for the occurrence of acquired cerebellar mutism in children is the complication accompanying posterior fossa surgery (8).

Figure 1. Diffusion MRI images of cerebellar parenchyma, thalamus and pons where ischemic areas were observed

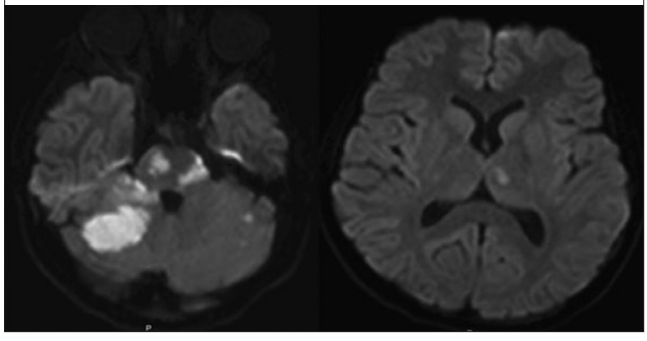
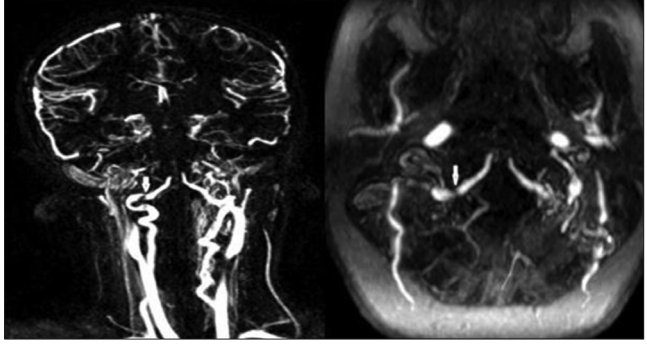


Figure 2. MR angiography, coronal and axial multiplanar reformat sequence where the vertebral artery was highly narrowed



The most important imaging methods for the diagnosis of CVD are MRI and MR angiography, with conventional angiography being the gold standard method (4). Also, MRI and MRA were employed for the diagnosis of our patient since no conventional angiography was performed. There is no general consensus on the treatment of VAD at present. Current treatment interventions include administration of antithrombolitic agents and anticoagulant agents (9). We administered the anticoagulant treatment to our patient. We commenced treatment with enoxiparin sodium and warfarin sodium administrations, and later continued with warfarin sodium only. Physical therapy is an important medical treatment for morbidity in diseases caused by cerebrovascular events. We commenced physical therapy as early as possible and, as a result, obtained a positive response on the patient. Although vertebral artery dissection is a rare disorder in childhood, it poses serious threats of mortality and morbidity (6). Our patient showed better response to clinical treatment when compared to previously reported cases in literature. Complete recovery was observed in aphasia after 45 days of treatment, as well as in left hemiparesis.

CONCLUSION

Taken together, VAD is a rarely diagnosed disease in childhood. Early diagnosis and treatment is very pertinent to reduce the mortality and morbidity of the disease. VAD should be investigated in cerebellar infarct cases, and angiography should be employed for the diagnosis.

Informed Consent: Written informed consent was obtained from the patient's family.

Peer-review: Externally peer-reviewed.

Author contributions: Concept – M.A., S.G.; Design – M.A., S.G.; Supervision – S.G.; Resource – B.Ö., S.K.; Materials – M.A., S.K.; Data Collection and/or Processing – B.Ö., S.K.; Analysis and/or Interpretation – M.A., S.G.; Literature Search -M.A., S.K.; Writing – M.A., B.Ö.; Critical Reviews – S.K., S.G.

Conflict of Interest: Authors have no conflict of interest to declare.

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Case Report

Transition of Pemphigus Vulgaris to Pemphigus Foliaceus Due to Non-Drug Substances

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ABSTRACT

Pemphigus is an auto-immune bullous disease which includes subgroups such as; Vulgaris, Foliaceus, and others. Pemphigus disease is characterized by bullous lesions and erosions of the skin and mucosae. The disease may develop due to the use of some drugs but sometimes it may flare up due to the misuse of some non-drug substances. We saw this in a 51-year-old patient who was advised by his charlatan friend to use a non-prescribed mixture which contained donkey milk, tar, puse, and tree root water. His pemphigus vulgaris disease showed the transition to foliaceus subtype accompanied by secondary erythroderma. When we scanned through literature, we noticed that this is the first case of pemphigus subtype conversion triggered by non-drug substances intake.

Keywords: Dermatitis, environment and public health, exfoliative, pemphigus

INTRODUCTION

Pemphigus includes a group of life-threatening bullous diseases of the skin and mucous membranes, characterized by flaccid bullae and erosions (1). The major subtypes of pemphigus are; pemphigus vulgaris (PV), pemphigus foliaceus (PF), pemphigus vegetans, and paraneoplastic (2). The transition from PV to PF has been reported in some few cases in literature (3). The use of alternative treatment methods by patients has become widespread today. Using oral and topical herbal supplements has increased recently (4, 5). Herein we reported the case of a patient who showed transition from PV to PF with characterized erythrodermic presentation as a results of the use of a mixture of donkey milk, tar, puse, and tree root water while he was being followed up with the diagnosis of PV.

CASE PRESENTATION

A 51-year-old male patient was examined due to erosion in bilateral pharyngeal arcus, bullae in the intermammary region and erosive plaques on the back. And the diagnosis of PV was made based on a histopathological examination (Figure 1) of the bulla on the dorsal region and direct immunofluorescent (DIF) examination of the perilesional area. We did not take a picture of the patient at that time because the clinical appearance and histopathological examination or DIF findings was typically consistent with PV. We started the patient on 80 mg methylprednisolone and 1440 mg mycophenolate mofetil, then the dose of methylprednisolone was gradually reduced in 2 years with regular monthly visits. The patient was followed up at the outpatient clinic. At the last state, he was in remission under treatment with 16 mg methylprednisolone and 1440 mg mycophenolate mofetil per day. He was clinically fine at that time and had no lesions or erosions.

The patient did not come for follow up visits for 4 months. And he told us he had used his drugs regularly as we mentioned above during that time interval. And at the same time, he said he had drunk a mixture of donkey milk, tar, puse, and tree root water three times a day for the last three weeks and the rash occurred 2 weeks after starting the mixture. He confessed that the mixture was not prescribed and that he bought it from his friend. This charlatan friend had persuaded our patient that the mixture was going to relieve his symptoms and cure him. Then the patient was admitted in our clinic with fever, widespread rash, and watery lesions. He said he had stopped the mixture after the rash started. But in 2 weeks the rash had already spread and was accompanied with secondary infection which lead to his coming to the clinic. During dermatological examination, crusted plaques and alopecia lesions were detected on his scalp. Yellowish crusted plaque and erosions were seen on his whole body. Flaccid bullae were seen on the legs and edema was seen on his scrotum and the penis (Figure 2). Nikolsky's sign was positive.

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Figure 1. Hematoxylin & eosin (x100) staining for identification suprabasal detachment

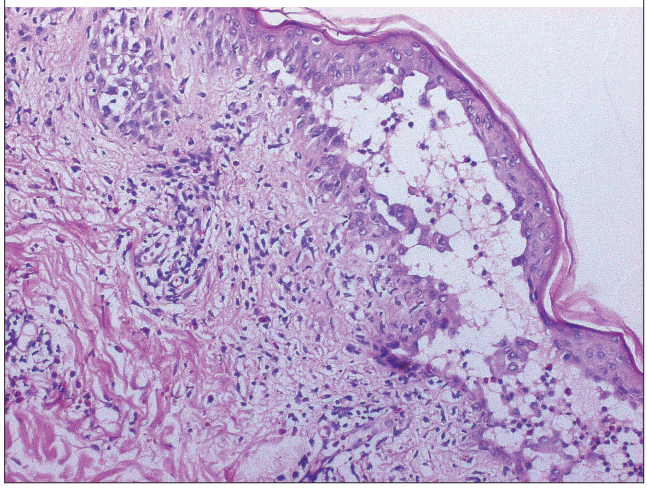
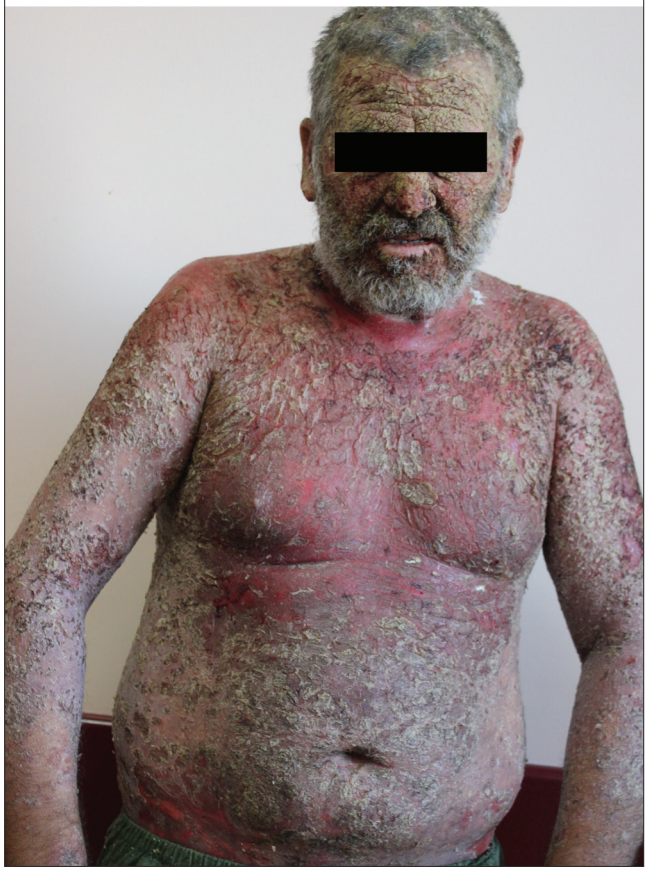


Figure 2. Crusted plaques and alopesic lesions were detected in the scalp, yellowish crusted plaques, erosions were detected in the whole body



Oral and genital mucosa had a normal appearance. A bilateral purulent discharge was seen in his eyes but the ophthalmologist did not observe any eye defect. His laboratory test results were as follows: WBC: 19.300/uL, platelet count: 704.000/uL, glucose: 262 mg/dL, total protein: 6.1 g/fL, albumin 2.5 g/dL, AST: 48 U/L,

Figure 3. Hematoxylin & eosin (x200) staining for identification subcorneal detachment and inflammatory cells

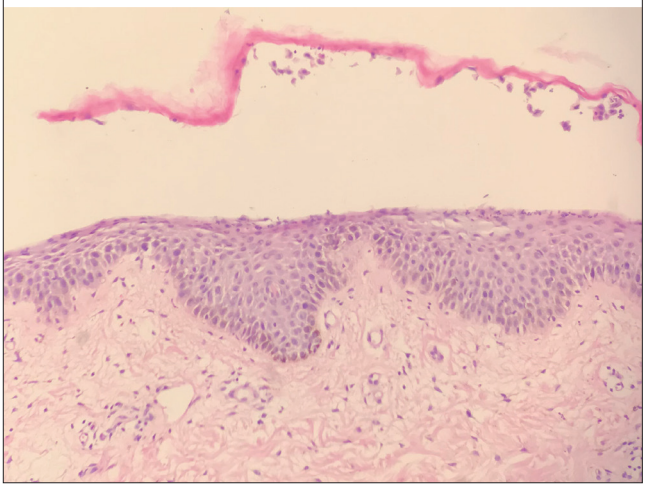
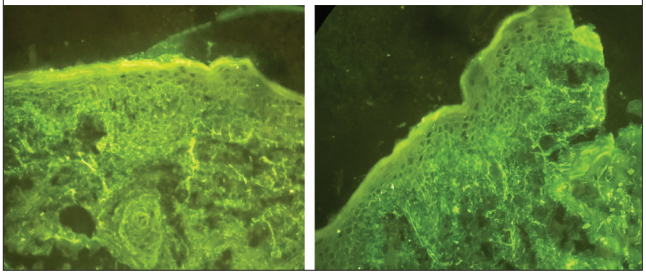


Figure 4. C3 and IgG accumulation in direct immunofluorescence (DIF) examination

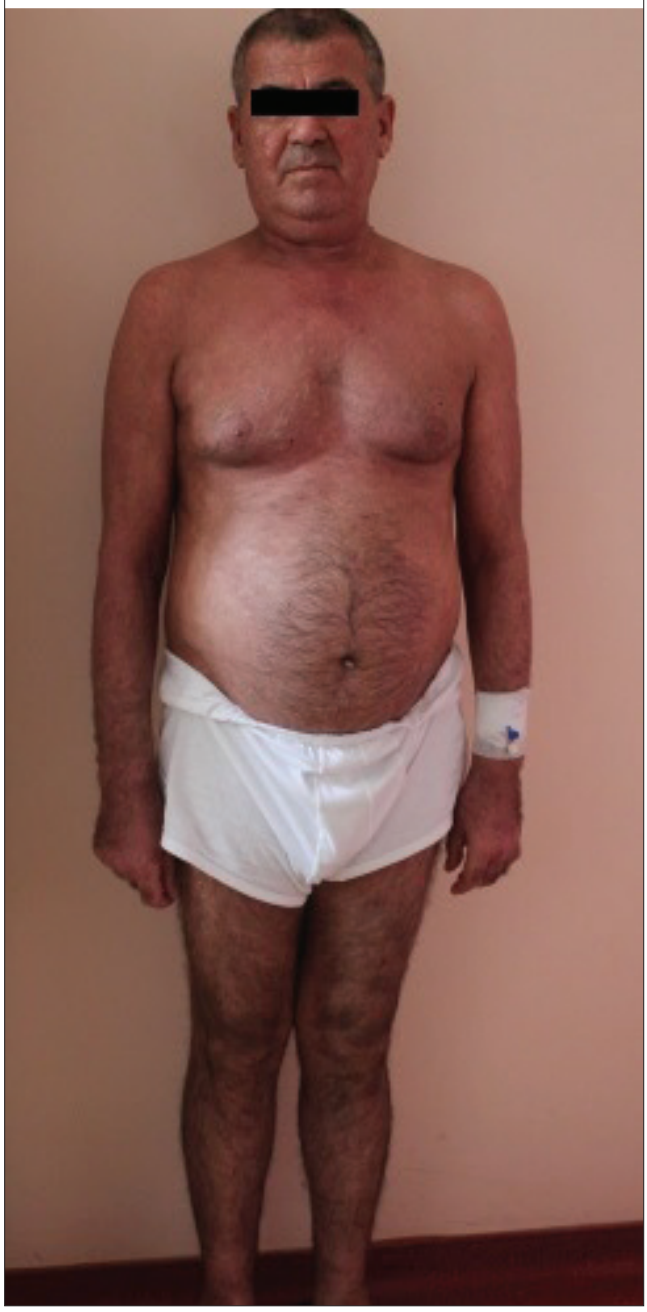


CRP: 51.7 mg/L, and electrolyte levels normal. When we tested for auto-immune markers (ANA, RF, TMAb, TGAb, etc.), they were all negative. So, we did not think there was any other accompanying auto-immune disorder in the patient. A sample for biopsy was taken from the bullae on the right arm and DIF examination from the perilesional area was done. Histopathological examination revealed subcorneal detachment and intraepithelial IgG, and a C3 accumulation was seen in the DIF examination (Figures 3 and 4). And the patient was diagnosed with PF based on these findings. The patient was then started on Methylprednisolone 120 mg and mycophenolate mofetil 1440 mg per day at the same time. With this treatment, new bullae development did not occur, current lesions regressed, and methylprednisolone dose was gradually reduced. The patient was discharged and scheduled for outpatient clinic controls with 40 mg methylprednisolone and 1440 mg mycophenolate mofetil as the lesions completely regressed (Figure 5). An oral informed consent was got from the patient.

DISCUSSION

The transition between PV and PF is rare and the etiopathogenetic mechanism of this situation is not known. This transition is proposed to be as a result of secondary auto-immune response resulting from the exposure of the immunologically hidden protein following the primary auto-immune or inflammatory pro-

Figure 5. The patient's eruption completely regressed after the treatment



cess which leads to tissue damage. This is defined as an epitope spread phenomenon. The transition from PF to PV following adrenal tumor and thymoma resection has been reported in literature (3).

The number of patients who have developed pemphigus after using herbal products is limited in literature. Dietary agents which are considered to trigger pemphigus include thiol compounds (garlic, leek, onion), phenols (black pepper, red pepper), tannins (tea, red wine, and spices), isothiocyanates (mustard, yogurt, cauliflower), and phytocyanins (spirulina paltenis alga). And the consumption of these substances are considered to trigger acantholysis (6-8). Donkey milk is a food supplement which contain proteins and angioedema was reported after its ingestion in one case (9). Information about each component of the mixture tar, puse, and tree root water is not available. The mechanism of action of these food supplements is not exactly known. Since the Nikolsky's sign was positive in our patient during clinical examination, we excluded a Toxic Epidermal Necrolysis (TEN). TEN or Lyell's syndrome is an acute dermatological emergency with significant morbidity and mortality. It characterized by bullous cutaneous lesions, exfoliations, erythematous maculae with a necrotic center, and serious mucosal erosions. Epidermal detachment on the body and mucosal involvement in two or more sites is observed. It is histopathologically characterized by full thickness necrosis (10). Subcorneal detachment is found in PF and it was important for differential diagnosis although accumulation in DIF is similar for both. Although this serious clinical manifestation is similar to TEN, we excluded TEN due to the lack of full thickness necrosis at histopathology and mild mucosal involvement in our patient. Clinical manifestations and histopathologic examination of our patient was consistent with erythrodermic PF.

Patients with dermatological diseases seek alternative treatments and this may result in life-threatening outcomes. We would like to underline that we had a limitation because we could not do the ELISA tests or IIF to detect antibodies due to the lack of the necessary devices. We did not have the opportunity to study the anti-Dg antibodies or IIF titre, because we did not have appropriate technical devices to do these tests. But in this case, we did the histopathological examination and DIF, and to the best of our knowledge the DIF test and histopathological findings are gold standards for the diagnosis of this disease, if the findings are consistent with clinical appearance.

CONCLUSION

When we scanned through literature, we noticed that this is the first case of pemphigus subtype conversion triggered by non-drug substances intake. We strongly advise that dermatologists should meticulously inquire the use of alternative treatment methods when there are changes in dermatoses form or when exacerbation occurs. The patients should also be informed not to use wrong alternative methods.

Informed Consent: Oral informed consent was obtained from patient who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - M.D., S.C., S.F., K.D.; Design - M.D., S.C., S.F., K.D.; Supervision - M.D., S.C., K.D.; Resource - M.D., S.C., S.F., K.D.; Materials - M.D., S.F., K.D.; Data Collection and/or Processing - M.D., S.C., S.F., K.D.; Analysis and/or Interpretation - M.D., K.D.; Literature Search - M.D., S.C., K.D.; Writing - M.D., S.C., S.F., K.D.; Critical Reviews - M.D., S.C., S.F., K.D.

Conflict of Interest: Authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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Case Report

Transverse Colon Volvulus: A Rare Cause of Ileus with Large Intestine–Origin

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ABSTRACT

Transverse colon volvulus is a disease that mostly occurs in the elderly and is rare in young people and children. Some of the predisposing factors for transverse colon volvulus are mental retardation, dysmotility disturbances, presence of narrow or long mesentery, and chronic constipation. One of the treatment options is endoscopic reduction, which is performed in patients whose overall condition is stable. It is not considered in case of emergency surgery. The success rate is low and the recurrence rate is high. Herein, we present the case of an 80-year-old female patient who was diagnosed with transverse colon volvulus as a rare cause of intestinal obstruction

Keywords: obstruction, volvulus, transverse colon

INTRODUCTION

Volvulus is when part of the intestine wraps around itself and its own mesentery resulting in a bowel obstruction. Transverse colon volvulus is one of the rare causes of intestinal obstruction. A successful treatment can be achieved with early diagnosis and timely intervention. The intraoperative presence of necrosis in the colon is the most important prognostic factor in these patients (1). In this article, we present the case of an 80-year-old female patient who was diagnosed with a transverse colon volvulus and was admitted due to complaints of severe abdominal pain and constipation.

CASE PRESENTATION

An 80-year-old female patient was admitted in the emergency department with complaints of abdominal pain and difficulty in defecation persisting since 4 days. The patient was morbidly obese with a history of heart failure. She had diffused abdominal tenderness and distention, and the auscultated intestinal sounds were hypoactive and partially metallic. Laboratory tests results were as follows: white blood cell: 20100/mm^3, hemoglobin: 9.5 g/dL, urea: 67 mg/dL, creatinine: 1.25 mg/dL, glucose: 139 mg/dL, albumin: 2.7 g/dL, sodium: 134 mmol/L, potassium: 3.2 mmol/L, calcium: 8.1 mg/dL, and c-reactive protein: 36 mg/dL. Other biochemical parameters were normal. Direct abdominal X-ray performed in a standing position showed dilated colonic segments and air-fluid levels. Computed tomography imaging showed that the transverse colon was dilated with intense colonic gas and perihepatic free fluid in the intestinal loops (Figure 1). No endoscopic detorsion procedure was performed. Based on the physical examination findings, an emergency laparotomy was decided. During exploration, the transverse colon was seen to be torsional, severely dilated, and edematous (Figure 2). It was seen that the peristaltism of the transverse colon was partially unclear, ischemic areas were seen, and there was no necrosis. The segment of the right colon proximal to the volvulus and the cecum were also dilated. Approximately 1000 cc of intraabdominal inflammatory reactional fluid was aspirated, and a subtotal colectomy, a terminal ileostomy, and a right hemicolectomy were performed. The patient was intubated and monitored in the postoperative intensive care unit and died on postoperative day 14 because of multiple organ failure.

DISCUSSION

Transverse colon volvulus is rarely seen compared to sigmoid and cecal volvulus. Abnormal rotation of the bowel results in a closed loop. This rotation takes place along the mesenteric axis, resulting in venous occlusion first and then arterial occlusion. The predisposing factors for transverse colon volvulus are excessive colon mobilization and chronic constipation (2).

Transverse colon volvulus has two different clinical presentations; acute fulminant or subacute progressive form. The acute form presents with complaints such as; sudden-onset severe abdominal pains, distention, abdominal tenderness, vomiting, leukocytosis, and a rapid worsening of the overall condition. Even though intestinal sounds are hyperactive at the beginning,

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Figure 1. Computed tomography image of the dilated transverse colon

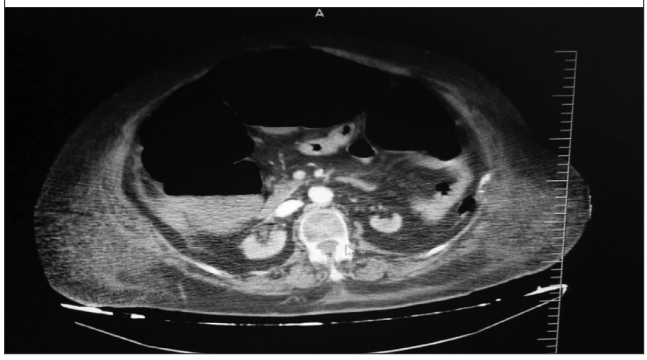
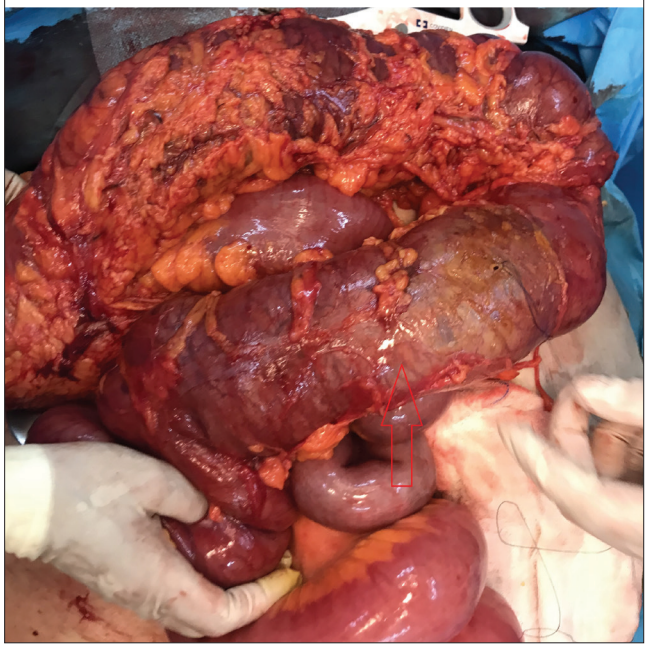


Figure 2. Intraoperative view of the torsioned and dilated transverse colon (red arrow)



they can disappear with time. In the subacute form, symptoms are unclear and intermittent. The severity of abdominal pain is less. Nausea and vomiting may not occur. Number of leukocytes may be normal or a little high. The lack of leukocytosis may be due to the lack of ischemia. Distention is more prominent than abdominal pain (3).

Preoperative diagnosis of transverse colon volvulus cannot be made normally. The colonoscopic detorsion procedure for treatment is usually considered as a temporary remedy before preparing the patient for an elective surgery. Hemorrhagic colonic content during colonoscopy is due to ischemia and strangulation. In case of unsuccessful detorsion and the presence of acute abdominal symptoms, surgery must be done immediately (4).

Sigmoid volvulus can be detorsioned by sigmoidoscopy or colonoscopy while a surgical operation is usually required for transverse colon volvulus. The surgical options are; detorsion, resection and primary anastomosis, detorsion through colopexy, resection and colostomy as well as mucous fistula, resection, anastomosis and deflector ileostomy. Detorsion and detorsion through colopexy have high rates of recurrence. The intraoperative method is decided taking into account the overall condition of the patient, intestinal contamination degree, intraabdominal contamination and ischemic situation of the colonic segments. The intraoperative presence of necrosis in the colonic wall is the most important prognostic factor for these patients (5).

In literature, it is emphasized that the most appropriate treatment procedure should be chosen based on the location of the volvulus and time of admission (6, 7). We did not consider doing an anastomosis in our case because of the risk of leakage as the proximal segment to the colon was excessively dilated, the complaints had persisted for 4 days, and the lack of preoperative intestinal preparations. We decided to do an ileostomy to the terminal ileum for protection purpose.

CONCLUSION

Even though transverse colon volvulus is a rare type of colonic volvulus, it should be kept in mind especially in elderly patients who are admitted due to signs of bowel obstruction.

Informed Consent: Oral informed consent was obtained from the son of the patient who participated in this study.

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