

Investigation of the relationship between insulin resistance and neuropeptide Y levels in polycystic ovary syndrome

Polikistik over sendromunda insülin rezistansı ve nöropeptid Y düzeyleri arasındaki ilişkinin araştırılması

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ABSTRACT

Objective: The aim of study is to investigate the relationship between neuropeptide Y (NPY) and insulin resistance which is important in the pathogenesis of polycystic ovary syndrome (PCOS).

Materials and Methods: This study was conducted between May 2012 and May 2013. The study included 45 patients with PCOS and 44 healthy controls at productive age. Insulin, fasting blood sugar, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, testosterone, dehydroepiandrosterone sulfate (DHEA-S), thyroid stimulating hormone (TSH), cortisol, estradiol, and NPY levels were measured at early follicular phase in patients with PCOS, while, insulin, fasting blood sugar, prolactin, DHEA-S, TSH, cortisol, and 17-OH progesterone levels were measured in control group. Homeostatic model assessment for insulin resistance (HOMA-IR) scores were calculated and antropometric measures were recorded. Pelvic ultrasonography was performed.

Results: Fasting insulin levels and HOMA-IR scores showed insulin resistance to be higher in obese patients with PCOS when compared with healthy controls and patients with normal weight PCOS. NPY levels were found to be higher in obese-overweight patients with PCOS than the values observed in healthy controls and in patients with normal weight but they were not statistically significant ($P>0.05$). NPY levels did not differ in patients with and without insulin resistance.

Conclusion: No correlation was dedected between insulin resistance and NPY levels but NPY levels were higher in overweight PCOS patients.

Keywords: Polycystic ovary syndrome, Insulin resistance, Neuropeptide Y

ÖZ

Amaç: Çalışmanın amacı, polikistik over sendromu (PKOS) patogeneğinde önemli yeri olan nöropeptid Y (NPY) ve insülin direnci arasındaki ilişkinin araştırılmasıdır.

Gereçler ve Yöntemler: Bu çalışma Mayıs 2012 ve Mayıs 2013 tarihleri arasında yapılmıştır. Çalışmaya doğurganlık çağındaki 45 PKOS hastası ve 44 sağlıklı kontrol grubu dahil edilmiştir. PKOS grubuna erken foliküler fazda insülin, açlık kan şekeri, folikül stimüle edici hormon (FSH), luteize edici hormon (LH), prolaktin, testosteron, dehidroepiandrosteron sülfat (DHEA-S), tiroid stimüle edici hormon (TSH), kortizol, östrodiol ve NPY düzeyleri bakılırken, kontrol grubuna insülin, açlık kan şekeri, prolaktin, DHEA-S, TSH, kortizol, 17-OH progesteron düzeyleri incelendi. Homeostatik model insülin direnci değerlendirme (HOMA-IR) testi skorları hesaplandı ve antropometrik ölçümler kaydedildi. Pelvik ultrasonografi yapıldı.

Bulgular: İnsülin direncini gösteren açlık kan insülin düzeyi ve HOMA-IR skoru, obez PKOS grubunda normal kilolu PKOS ve kontrol grubuna göre yüksek bulundu. NPY düzeyleri obez-yüksek kilolu PKOS grubunda normal kilolu PKOS ve kontrol grubuna göre yüksek bulundu ancak istatistiksel olarak anlamlı değildi ($P>0,05$). NPY düzeyleri insülin rezistansı olan ve olmayan hastalar arasında değişiklik göstermedi.

Sonuç: İnsülin rezistansı ve NPY düzeyleri arasında korelasyon saptanmadı ancak NPY düzeyleri, kilolu PCOS hastalarında yüksekti.

Anahtar kelimeler: Polikistik over sendromu, İnsülin rezistansı, Nöropeptid Y

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women during reproductive period and characterized by hyperandrogenism, chronic anovulation and polycystic over appearance on ultrasonography (US)

[1]. Insulin resistance and hyperinsulinemia can be observed in both weak and overweight/obese PCOS patients, as an important feature [2]. However, insulin resistance is not a necessary parameter for the diagnosis of PCOS. The effect of insulin resistance in PCOS has not yet been completely elucidated, but insulin resistance is thought to play an important role in the pathogenesis of PCOS. It has been shown that insulin resistance in PCOS is caused by some molecular disorders that occur in the post-receptor insulin signalling pathway.

Neuropeptide Y (NPY) is an appetite enhancing peptide and its effect on appetite was first shown in 1984. It is structurally and immunologically similar to the pancreatic polypeptide, belongs to the pancreatic polypeptide family, and consists of 36 amino acids [3]. NPY is commonly found in the central and peripheral nervous system. In many studies, in which NPY has been applied to the central nervous system, it has been shown that NPY regulates nutrient intake and body weight. In addition, it has been demonstrated in studies that NPY has an inhibitor effect on the hypothalamo-hypophyseal-ovarian axis [4]. This study aims to determine the relationship between insulin resistance and NPY levels in patients with PCOS for the etiopathogenesis.

Material and Methods

This study was carried out prospectively and controlled between May 2012-May 2013. A total of 45 women, 25 normal weight (Body mass index (BMI) <25kg / m²) and 20 overweight or obese (BMI> 25kg / m²) with PCOS who in reproductive period were included in the study. For the control group, 44 healthy women without any chronic disease, menstrual irregularity and hyperandrogenemia were included. Ethical committee approval has been obtained before commencement of the study (No: 2012/202). The participants were informed about the purpose of the study and the procedures. "The Androgen Excess and PCOS Society Criteria" were considered as a diagnostic criteria in the selection of PCOS patients [5]:

1. Hirsutism and / or hyperandrogenemia,
2. Oligo/anovulation and / or polycystic ovaries,
3. Exclusion of other causes.

"Ferriman-Gallwey Score" was used for hirsutism scoring. Patients with a score of 8 and above were included into the study group. Patients with any systemic disease, androgen-releasing tumor or hyperprolactinemia, drug users (oral contraceptives,

metformin, glitazones, NSAID) that affect insulin resistance, and smokers were not included into the study.

Patients BMI and waist / hip ratios were calculated. Serum levels of blood glucose, insulin, NPY, total testosterone, follicular stimulating hormone (FSH), luteinizing hormone (LH), 17-OH progesterone, prolactin (PRL), dehydroepiandrosterone sulfate (DHEA-S) and thyroid stimulating hormone (TSH) were measured in all patients at the early follicular phase (2-5 days of menstruation) after 12-hour fasting.

NPY levels were studied using enzyme-linked immunosorbent assay (ELISA) method using USCN brand (USCN Life Science Inc., E90879Hu, 96 Tests, China) kits. The measurement range of the method was 2.47-200 pg / mL. Glucose level was studied in the Cobas Integra 800 autoanalyser (Roche Diagnostics, Mannheim, Germany). Insulin, cortisol, testosterone, FSH, LH, estrogen, prolactin, DHEAS, TSH, free T4 and T3 levels were studied by the electrochemiluminescent method in the Modular E170 autoanalyser (Advia Gentaur XP Siemens). 17-OH Progesterone levels were determined by ELISA method using the 17-OH Progesterone ELISA (DSX automated ELISA SYSTEM) commercial kit. HOMA-IR was calculated by $[\text{Fasting Insulin (mIU / ml)} \times \text{Fasting blood glucose (mg/dl)}] / 405$ formula. For insulin resistance, HOMA-IR values of 2.5 and above were accepted.

Ultrasonography measurements of the patients were made by using transabdominal US. According to the 2003 Rotterdam consensus, a polycystic over-view was defined when there were 12 or more follicles 2-9 mm in diameter and / or the ovary volume was above 10cm [3].

Statistical Analysis

The normal distribution suitability of the variables was examined by the Shapiro Wilk test. Variables that provided normal distribution assumption were summarized in terms of mean \pm SD, while variables that did not provide assumption were summarized as median [min-max]. Categorical variables were expressed in numbers and percentages. The independent sample t test was used when the distribution assumption was provided in the comparison of the two groups, whereas the Mann Whitney U test was used when the assumption is not provided. ANOVA was used when the distributional assumption was provided in more than two groups comparisons and Tukey was used as a post hoc test. In case of not provided, it was analyzed by Kruskal Wallis test and Dunn test was applied as post hoc test. The Spearman correlation coefficient

was calculated to determine the relationship between the two continuous variables. Statistical significance was $P < 0.05$. The SPSS 11.5 program was used in the data analysis.

Results

Forty-five female patients and 44 healthy volunteer women were included in the study. Demographic and biochemical characteristics of the patient and control groups are shown in Table I.

Hirsutism scores, insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) levels in patients with PCOS were higher and statistically significant than control group ($P < 0.05$). No statistically significant difference was found between the PCOS group and the control group in terms of other parameters ($P > 0.05$).

Table I. Demographic and biochemical characteristics of the patient and control groups

	PCOS	Control	P
Age (year)	24.89±5.17	26.02±3.41	0.051
Height (cm)	163.09±6.81	161.66±4.30	0.239
Weight (kg)	68.97±18.85	62.67±11.26	0.139
BMI (kg/m ²)	25.71±6.07	23.90±3.98	0.205
WS (cm)	79.93±13.66	76.22±9.49	0.216
HS (cm)	101.69±15.02	98.53±11.42	0.406
W / H (cm)	0.78±0.04	0.77±0.04	0.222
HS	12.24±1.88	3.66±0.80	<0.001
FBG (mg/dl)	91.21±6.89	87.58±5.76	0.214
IL (mIU/ml)	9.26±6.10	4.68±4.12	<0.001
HOMA-IR	2.13±1.46	0.98±0.88	<0.001
NPY pg/mL	82.38±74.71	59.25±55.03	0.080

(BMI: Body mass index, WS: Waist size, HS: Hip size, W/H: Waist / Hip Ratio, HS: Hirsutism score, FBG: Fasting blood glucose, IL: Insulin level, HOMA-IR: Homeostatic model assessment for insulin resistance, NPY: Neuropeptide Y)

Patient and control groups were divided into two subgroups with normal BMI and high BMI. The demographic characteristics, HOMA-IR, insulin and NPY levels of the PCOS and control subgroups are shown in Table II. All findings in the overweight-obese PCOS subgroup were higher and statistically significant than the normal weight PCOS group ($P < 0.001$). In control subgroups, waist and hip ratios, insulin levels and HOMA-IR values were found to be higher and statistically significant in overweight - obese subgroups when compared to the normal weight control group ($P < 0.05$). No statistically significant difference was found in the comparison of the other data in the control group.

There was a statistically significant difference between the overweight PCOS group and the control group for all three findings (fasting blood glucose (FBG), insulin, HOMA-IR) when compared with the findings of PCOS subgroups and control group. There was a significant difference for FBG between overweight PCOS and normal PCOS groups but there is no significant difference in comparison of other findings. The difference between the insulin level and insulin resistance of the patient and control groups was also statistically significant ($P < 0.001$).

No statistically significant results were found in the patient and control groups when the relationship was evaluated between insulin and NPY levels, BMI and NPY levels, waist size and NPY levels (Table III).

Values above 2.5 of HOMA-IR indicate insulin resistance at varying degrees and insulin resistance was found in 48.8% of the patients in the subgroup of PCOS, and 9% of the control group. The insulin level of patient group was found to be statistically significantly higher than the control group ($P < 0.05$). In PCOS patients with insulin resistance, NPY

Table II. Demographic characteristics, HOMA-IR, Insulin and NPY values of PCOS and control subgroups

	PCOS		P	Control		P
	BMI <25	BMI ≥25		BMI <25	BMI ≥25	
	mean±SD	mean±SD		mean±SD	mean±SD	
Age (year)	22.92±4.07	26.85±5.43	0.142	25.00±2.60	27.00±3.98	0.213
Height (cm)	162.56±7.50	163.75±5.95	0.566	161.57±4.39	161.81±4.26	0.860
WS (cm)	71.56±7.75	90.40±12.20	<0.001	71.13±5.90	84.81±8.16	<0.001
HS (cm)	93.04±8.28	112.50±14.64	<0.05	93.04±7.81	105.50±10.65	<0.001
W / H	0.77±0.04	0.80±0.04	<0.05	0.76±0.04	0.79±0.03	0.059
IL (mIU/L)	6.88±6.44	12.25±4.12	<0.001	3.30±1.61	7.26±5.92	<0.05
HOMA-IR	1.54±1.50	2.86±1.05	<0.001	0.70±0.34	1.47±1.26	<0.05
NPY (pg/ml)	71.08±60.66	96.50±88.89	0.451	56.57±54.08	63.94±58.14	>0.05

(WS: Waist size, HS: Hip size, W/H: Waist / Hip ratio, IL: Insulin level, HOMA-IR: Homeostatic model assessment for insulin resistance, NPY: Neuropeptide Y)

levels were found to be higher than patients without insulin resistance but it was not statistically significant (P: 0.595). There was no statistically significant difference when NPY levels were compared between obese and normal weight PCOS patients and control group (Overweight PCOS-control, normal weight PCOS-control and overweight PCOS-normal weight PCOS P values were 0.094, 0.751, 0.451, respectively).

Table III. The relationship between insulin and NPY levels, BMI and NPY levels, waist size and NPY levels in the patient and control groups

	PCOS (n:45) Patient	PCOS (n:44) Control
Insulin and NPY levels – r	0.003	0.086
Insulin and NPY levels – P	0.983	0.581
BMI and NPY levels – r	0.031	0.119
BMI and NPY levels – P	0.838	0.440
Waist size and NPY levels – r	0.148	0.164
Waist size and NPY levels – P	0.332	0.292

Discussion

Polycystic ovary syndrome is the most common endocrine disorder in women during reproductive age [1]. The disease presents with chronic anovulation and hyperandrogenism findings [6]. Despite many studies, the pathogenesis of PCOS is still unknown completely, but it is a fact that insulin resistance and hyperinsulinemia play an important role in the pathogenesis of the disease [7-11]. It has been shown that women with PCOS have more insulin resistance and hyperinsulinemia than normal women who are in similar age and weight [12-14]. The relationship between glucose intolerance and hyperandrogenemia was first described by Achard and Thiers in 1921 and is called “diabetes in bearded women” [2-15]. The incidence of insulin resistance in patients with PCOS ranges from 53% to 75% [13,16,17]. Various methods can be used to demonstrate insulin resistance, one of which is the HOMA-IR method [15]. Values 2.5 and above according to the HOMA-IR method reflect insulin resistance. In our study, insulin resistance in patients with PCOS was statistically significantly higher than the control group. When the PCOS group was divided into two groups as normal weight and overweight-obese, the HOMA-IR level of overweight – obese PCOS group was statistically higher than normal weight PCOS group. On the other hand, some studies have shown that PCOS patients with normal weight have insulin resistance, while some of them show that insulin resistance is absent [13,14,17,19].

There are studies showing that insulin resistance is more severe in overweight – obese PCOS patients [20]. In our study, the mean insulin resistance in the overweight-obese group was also higher than the normal weight PCOS group.

Patients with PCOS have a 50-60% obesity rate [21]. Clinical and hormonal disorders become more prominent in PCOS patients with obesity. Metabolic and endocrine parameters are improved in these patients with weight loss [22,23]. Patients with overweight-obese PCOS usually have android type fat distribution [24,25]. In our study, the waist size and waist / hip ratio in overweight-obese PCOS patients were also significantly higher than normal women. This findings suggest that our overweight-obese PCOS patients have an android-like body fat distribution. On the other hand, there are studies in the literature that have different results regarding the occurrence of android type fat distribution in PCOS patients with normal weight [24-27]. The presence of android type fat distribution in patients with PCOS has been reported as a good indicator of insulin resistance and metabolic disorders [28,29].

Neuropeptide Y is an appetite enhancing peptide and its effect on appetite was first shown in 1984 [27]. Insulin and leptin cause a decrease in NPY levels, while ghrelin and glucocorticoid lead to an increase [30,31]. NPY also has an inhibitor effect on the hypothalamo-hypophyseal-ovarian axis [32]. Studies have shown that NPY neurons in the pancreas regulate insulin secretion, and also hyperinsulinemia and insulin resistance develop after prolonged exposure to NPY [32-35]. In our study, no statistically significant relationship was found between insulin resistance and NPY levels in patients with PCOS. However, in the group of PCOS patients with insulin resistance, the NPY level was found to be higher than the group with non-insulin resistant PCOS.

Neuropeptide Y values were found to be high in patients with obese and non-obese PCOS in a study conducted by Baranowska et al. [36]. On the other hand, it was shown that, NPY values increased in obese women without PCOS as BMI values increased. In the study of Baranowska et al., there was no relationship between NPY and insulin levels. But, in our study, the NPY levels in PCOS patients were higher than the NPY levels of the control group, but these findings were not statistically significant. The NPY level in the overweight-obese PCOS group was higher than the NPY level of the normal weight PCOS group, but this finding was not statistically significant. Furthermore, contrary to the study of Baranowska et al., in our study, no statistically

significant finding was found between BMI and NPY level in the control group. This difference may be due to the low number of overweight – obese patients in the control group of our study. Also, similar to this study, there was no relation between insulin and NPY level in our study.

In the study of Gunes and Bukan, the patients were divided into three groups as obese PCOS, normal weight PCOS and control groups. According to this study, in obese PCOS patients, NPY levels were higher than other groups and they were statistically significant [37]. In our study, NPY levels in obese PCOS patients were higher than normal weight PCOS patients and control group, but it was not statistically significant.

In a study by Orbetzova et al., that included non-PCOS overweight-obese and normal weight patients, NPY levels were found to be higher in normal weight patients [38]. Orbetzova et al., declared that the lower NPY levels of the obese patients comparing to control group, may be due to increased levels of leptin. In our study, although, the NPY levels of overweight – obese control group were higher than the NPY levels of normal weighted group, there was no statistical difference. This result was found to be opposite to the findings of Orbetzova et al. [38].

The most common sign of hyperandrogenism in PCOS is hirsutism and is evaluated with modified Ferriman-Gallwey method [39]. In our study, we used the Ferriman Gallwey scoring method and hirsutism score was found to be higher in PCOS patients compared to the control group and it was statistically significant.

Some studies on the relationship between NPY levels and BMI in PCOS patients have shown different results. Baranowska et al. detected that the NPY level in overweight-obese PCOS patients was lower than the overweight obese – control group [36]. In the study of Gennarelli et al., NPY levels were close to each other in the overweight-obese PCOS and overweight-obese control group, but the impaired response of NPY with hypoglycemia was observed [40]. In our study, NPY levels were found to be higher in the overweight-obese PCOS group than the NPY levels of the overweight-obese control group, in contrast to the findings of both two studies. This difference is thought to be due to the numbers of patients taken into the study are different.

In conclusion, NPY level was higher in obese and normal weight PCOS patients than control group but it was not statistically significant. In addition, NPY levels in patients with insulin resistance were higher than PCOS

patients without insulin resistance, but it was not statistically significant. We think that multicenter studies with more patients are needed to reveal these relationships.

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Evaluating adherence to long-term prophylaxis treatment with danazol in adult hereditary angioedema patients: A real life study

Erişkin herediter anjiödem hastalarında danazol ile yapılan uzun dönem profilaksi tedavisine uyumun değerlendirilmesi: Gerçek bir yaşam çalışması

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ABSTRACT

Objective: To investigate the adherence to the prophylactic treatment in hereditary angioedema (HAE) patients as well as the potential factors which may affect this situation.

Patients and Methods: In addition to evaluation of their medical records, sixty HAE patients were asked to complete a questionnaire including inquiries about demographic and clinical features of their disease and medications used. Disease severity was determined depending on their age of onset of symptoms, clinical manifestations, and need of long-term prophylaxis.

Results: Sixty-five percent of the patients were female, the mean age was 38.07±12.38 years, 93.3% were type 1 HAE, 58.3% had a severe form of the disease, and 71.7% were under prophylaxis with danazol. Fourteen patients were not using danazol regularly due to the fear of side effects (n=11) and forgetfulness to take the medication (n=4). It was observed that the patients who were the only cases in their families, those having few relatives with HAE and having had no excitus due to HAE in their families, were more adherent to prophylactic treatment (P=0.008; P=0.018; P=0.028).

Conclusion: The majority of patients were effectively under long-term prophylaxis and the majority adhered to this treatment. The primary cause of non-adherence was fear of side effects.

Keywords: Adherence to treatment, Hereditary angioedema, Management, Long-term prophylaxis, Danazol, Side effects

ÖZ

Amaç: Herediter anjiödem (HAÖ) hastalarında profilaktik tedaviye uyumu ve bunları etkileyen olası faktörleri araştırmaktır.

Hastalar ve Yöntemler: Altmış HAÖ hastasının tıbbi kayıtları değerlendirildi ve demografik ve hastalıkları ile ilgili klinik özellikler ve kullandıkları ilaçlar ile ilgili soruları içeren anketi yanıtlamaları istendi. Hastalık ciddiyeti, semptomların başlangıç yaşı, klinik bulguları ve uzun dönem profilaksi ihtiyacına göre belirlendi.

Bulgular: Hastaların %65'i kadın, yaş ortalaması 38,07±12,38, %93,3'ü tip 1 HAÖ, %58,3'ünün hastalığı ciddi idi ve %71,7'si danazol ile profilaktik tedavi görüyordu. Ondört hasta profilaktik tedavisini düzenli kullanmıyordu ve kullanmama nedenleri yan etki gelişmesinden korkmak (n=11) ve ilacı almayı unutmak idi (n=4). Ailede tek vaka olanların, HAÖ'li akrabası az olanların ve ailesinde bu nedenle hayatlarını kaybeden akrabası olmayanların profilaktik tedaviye daha uyumlu olduğu görüldü (P=0,008; P=0,018; P=0,028).

Sonuç: Hastalarımızın büyük çoğunluğu profilaktik tedaviyi düzenli kullanmakta ve bu tedaviden fayda görmektedir. Uyumsuzluğun primer nedeni yan etki gelişimi korkusudur.

Anahtar kelimeler: Tedaviye uyum, Herediter anjiödem, Tedavi, Uzun dönem profilaksi, Danazol, Yan etkiler

Introduction

Hereditary angioedema (HAE) is an orphan disease that develops due to the mutations in the SERPING1 gene. Mutations lead to deficiency (Type 1 HAE) or dysfunction (Type 2 HAE) in C1-esterase inhibitor (C1-INH) protein [1]. The estimated prevalence of the disease ranges from 1/10.000 to 1/100.000 and is autosomal dominantly inherited [1,2]. The deficiency or dysfunction in C1-INH protein causes overproduction of bradykinin which in turn leads to increment of vascular permeability by affecting the bradykinin 2-receptors on endothelial cells [2,3]. This phenomenon clinically results in edema attacks in mucocutaneous tissues

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such as the face, the larynx, the gastrointestinal tractus, the genitalia, and the extremities [2]. The episodes in HAE are characterized by nonerythematous, nonpruritic and well-demarcated swellings without urticarial lesions and/or abdominal pain [2]. The attacks can last 2-5 days and untreated larynx edema can result in death [2,4]. Accurate and early diagnosis is therefore very important in proper management of the disease. Unfortunately, misdiagnosis is common, causing a delay in diagnosis and mistreatment, even unnecessary surgical interventions because of physician unawareness [2,5,6].

The management of the disease includes avoidance of attacks with long or short term prophylaxis and treatment of acute attacks [1,7]. Plasma derived C1-inhibitor (pdC1-INH) concentrates, the bradykinin-2 reseptor antagonist (icatibant) and kallikrein inhibitor (ecallantide) are used to treat acute attacks [1,8] For patients who have frequent and severe attacks and those who do not have easy access to C1-INH concentrates, long-term prophylaxis is recommended [1].By means of long-term prophylaxis using attenuated androgens, pdC1-INH and anti-fibrinolytics, frequency, duration and severity of episodes can decrease significantly [1,9]. Acute angioedema episodes lead to direct medical costs as well as reduced performance and/or absenteeism at work and school [10]. Moreover, since the attacks are imponderable, patients are negatively affected regarding quality of life issues and become depressive in the long term [11-13]. The prevention of acute attacks is therefore of outmost importance to improve patient quality of life. In chronic illnesses which need long-term drug usage, adherence to treatment can be a matter for both phsicians and patients.

Noncompliance with medication causes decreased efficacy and treatment failure as well as increased medical costs [14,15]. In our country, plasma derived C1-INH concentrates are not authorized for prophylaxis and the only available attenuated androgen used for the prophylactic treatment of adult HAE patients is danazol. However, insufficient data exists regarding the adherence rate of HAE patients to this prophylactic treatment.

We aimed to evalaute the adherence rate to the long-term prophylactic treatment with danazol in HAE patients and the potential factors which may affect this adherence.

Patients and Methods

We conducted an observational and retrospective study. The data were mainly collected from the medical records of the HAE patients followed in our adult Immunology and Allergy Clinic. Additionally, the patients were requested to complete a questionnaire, including various inquiries regarding demographic and clinical features, when they came for routine visits.

Patients were classified as adherent or nonadherent depending on the regularity of their adherence to prescribed prophylactic treatment. Nonadherence was defined as skipping at least twice the two or more consecutive recommended doses of the drug, depending on the half life of danazol, i.e, approximately 24 hours. Most of our patients use danazol once or rarely twice per day. Demographic and clinical features were compared between these two groups.

Disease severity was assessed with the general disease severity score developed by Bygum et al. This score ranges from 0 to 10 points (10 is the highest disease severity), does not consider any specific time and appraises the entire course of the disease from symptom onset to evaluated time (Table I) [16]. Score lower than 7 was accepted as mild to moderate disease; score of 7 or more was considered as severe disease [17].

To evaluate the efficacy of long-term prophylactic treatment, the frequency of attacks was compared before and after the initiation of treatment.

Ethical approval for this study was obtained from the Istanbul University, School of Medicine Ethical Committee (Number:753/ID:2016/735) and written informed consent was obtained from all patients.

Table I. Clinical severity score (cumulated 0-10 points) [16]

Feature	Points
Age at onset 0-5 years	3
Age at onset 6-10 years	2
Age at onset 11-20 years	1
Age at onset >20 years	0
Skin edema ever	1
Painful abdominal edema ever	2
Laryngeal edema ever	2
Other clinical manifestations	1
Long term prophylaxis ever	1

Statistical analysis

The results were expressed as percentages and mean \pm standard deviation. The categorical and continuous variables were compared with chi square or Fisher's exact tests and independent Sample t Test or Mann-Whitney U test according to the distribution as normal or not. The frequency of attacks before and after long-term prophylaxis treatment was compared with Wilcoxon Signed Rank test. A 'P' value lower than 0.05 was accepted as significant.

Results

Sixty patients answered the questions. Sixty-five percent of the patients were female and the mean age was 38.07 ± 12.38 years. The mean general disease severity score was 6.7 ± 1.63 points and 58.3% of the patients (n=35) had severe disease. Most patients were type 1 HAE (93.3%) and 4 (6.7%) patients were type 2 and 71.7% (n=43) of the patients were under prophylaxis mostly with danazol. Only one patient used tranexamic acid. The mean duration of follow up under danazol was 63.51 ± 35.1 months (min-max:6-140 months). In 12 patients danazol use was discontinued due to pregnancy (n=8), side effects involving secondary amenorrhea (n=2) and hypertension (n=1), and inefficacy (n=1). Fourteen patients were not using the prophylactic treatment regularly due to fear of development of side effects (n=11) and forgetfulness (n=4). The mean age at development of first symptoms, age at diagnosis, and delay in diagnosis were 12.48 ± 9.45 , 30.05 ± 13.59 and 17.02 ± 12.95 years, respectively. Nearly half (56.7%) of the patients were misdiagnosed until the correct diagnosis was completed. In 13.3% of the patients, laparotomy surgeries (mainly appendectomy) were performed during acute abdominal attacks. A quarter (23.3%) of the patients were the sole cases in their families and 15% of the patients had lost their first degree relatives due to asphyxia. Detailed information about demographic and clinical features of the patients is shown in Table I. The frequency of the HAE attacks

significantly decreased after treatment in patients who were under long-term prophylaxis as shown in Figure 1 ($P < 0.001$).

Comparison of the demographic and clinical features of the adherent and nonadherent groups is given in Table II. It is observed that the patients who were the only cases in their families, those having less relatives with HAE, and ones with no excitus due to HAE in their families were more adherent to the prophylactic treatment ($P=0.008$; $P=0.018$; $P=0.028$). The frequency of abdominal pain attacks were significantly lower in adherent group than nonadherent group and although, the frequencies of other types of attacks were less in adherant group there were no statistically significant differences between the groups. The mean dose of danazole was 100 mg/day in both groups.

There were no correlation between disease severity and some factors such as age, gender, experiencing prodromal symptoms, having triggering factors in attacks, being a single patient and having excitus in their families. Also, disease severity did not affect the adherence to prophylaxis.

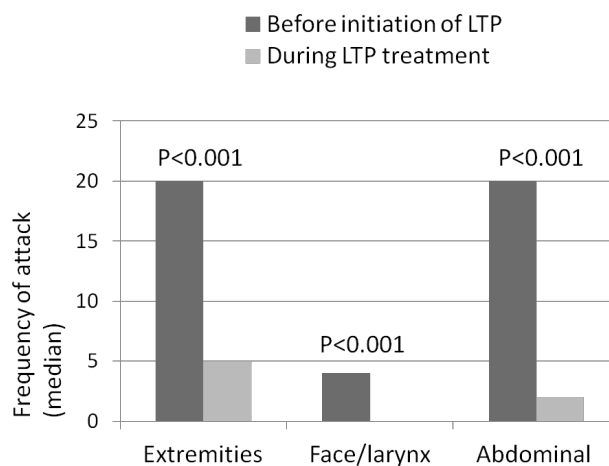


Figure 1. The frequencies of HAE attacks according to the site of attacks and before or during long-term prophylaxis treatment (LTP: Long-term prophylaxis)

Table II. Demographic and clinical features of all patients and the comparison of these features with the adherent and nonadherent patients who are under long - term prophylaxis treatment

	All patients n (%)	Adherent to prophylactic treatment n (%)	Not adherent to prophylactic treatment n (%)	P
Female gender	25 (65)	18 (62.1)	7 (50.0)	NS
Symptoms developed ever				
Abdominal pain	58 (96.7)	28 (96.6)	14 (100)	NS
Angioedema on face	48 (80)	25 (86.2)	13 (92.9)	NS
Laryngeal angioedema	41 (68.3)	21 (72.4)	11 (78.6)	NS
Angioedema on extremities	57 (95)	27 (93.1)	14 (100)	NS
Misdiagnosis before diagnosis of HAE	34 (56.7)	19 (65.5)	8 (57.1)	NS
Allergy	12 (20)	7 (24.1)	3 (21.4)	NS
Familial Mediterranean Fever	14 (23.3)	8 (27.6)	3 (21.4)	NS
Rheumatologic diseases	3 (5)	2 (6.9)	0	NS
Colitis	4 (6.7)	2 (6.9)	1 (7.1)	NS
Prodromal symptoms	44 (73.3)	19 (65.5)	12 (85.7)	NS
Lassitude/Fatigue	20 (33.3)	7 (24.1)	7 (50.0)	NS
Nausea	14 (23.3)	4 (13.8)	4 (28.6)	NS
Tingling	12 (20)	3 (10.3)	5 (35.7)	NS
Pain	6 (10)	2 (6.9)	2 (14.3)	NS
Irritability	3 (5)	1 (3.4)	2 (14.3)	NS
Other	46 (60)	7 (23.8)	8 (56.8)	NS
Triggering factor	56 (93.3)	26 (89.7)	14 (100)	NS
Stress	49 (81.7)	22 (75.9)	14 (100)	NS
Fatigue	23 (38.3)	7 (24.1)	7 (50)	NS
Trauma	42 (70)	19 (65.5)	12 (85.7)	NS
Hormonal	6 (26.7)	10 (34.5)	2 (14.3)	NS
Infections	14 (23.3)	10 (34.5)	3 (21.4)	NS
Minor/major surgical interventions	10 (16.7)	6 (20.7)	4 (28.6)	NS
Drug	9 (15)	4 (13.8)	3 (21.4)	NS
Apandectomy	8 (13.3)	5 (17.2)	1 (7.1)	NS
In family				
Being single case	14 (23.3)	11 (37.9)	0	0.008
Exitus due to this disease	9 (15)	2 (6.9)	5 (35.7)	0.028
Attack treatment				
To increase the dose of danazol	7 (11.7)	4 (13.8)	3 (21.4)	NS
C1 inhibitor concentrate	57 (95)	27 (93.1)	13 (92.9)	NS
Icatibant	17 (28.3)	7 (24.1)	3 (21.4)	NS
Fresh frozen plasma	2 (3.3)	1 (3.4)	0	NS
	Mean±SD/median	Mean±SD/median	Mean±SD/median	P
Age (year)	38,07±12,38	38,3±12.2	39,4±13.12	NS
Age of onset of symptoms (year)	12.48± 9.45	10	8.5	NS
Age of diagnosis (year)	30.05± 13.59	29.2±14.5	32.43±12.6	NS
Time interval between onset of symptoms and diagnosis (year)	17.02± 12.95	18.5±13.8	19±14.79	NS
Number of patients in the family	2	2	4	0.018
Abdominal pain attacks in a year before diagnosis/treatment	21.77± 16.0	20	22.5	NS
Attacks of angioedema on face and/or larynx in a year before diagnosis/ treatment	8.98± 11.72	3	4.5	NS
Attacks of angioedema on extremities in a year before diagnosis/treatment	26.22± 21.48	20	22.5	NS
Abdominal pain attacks in a year after diagnosis/treatment	7.4± 7.9	2	5.5	0.031
Attacks of angioedema on face and/or larynx in a year after diagnosis/ treatment	2.9± 5.12	0	0.5	NS
Attacks of angioedema on extremities in a year before diagnosis/treatment	9.12± 9.1	5	7.5	NS
Dose of danazol (mg)	100	100	100	NS
Number of C1 inh concentrates which were consumed in the last year	3	2	3	NS

Discussion

This study, to the best of our knowledge, is the first and sole study that evaluates the adherence to long-term danazol prophylaxis of HAE patients living in a developing country where the prophylactic treatment with plasma derived C1-INH is not licensed.

One of the main findings of the study is that most patients were under long-term prophylaxis (71.7%). The usage of long-term prophylaxis was higher in our study than the cohorts of the other countries in which it ranged from 23.2% to 56.3% [5, 11, 17-21]. As in our study, the most commonly used agent for long-term prophylaxis was attenuated androgen, namely danazol [11,18-20]. Attenuated androgens leading to an increase in plasma levels of C1-INH are useful agents in HAE patients to prevent acute attacks [22,23]. However, these androgens can cause adverse events involving liver toxicity and androgenic changes such as hirsutism and lipid profile disturbances; consequently careful follow-up is necessary in patients using these agents long-term [24,25]. Furthermore, their usage is contraindicated during pregnancy and breast feeding and is problematic until puberty [25,26]. These problems could explain the propensity to avoidance of adherence to attenuated androgens in both patients and physicians. Currently, in most European countries, pdC1-INH concentrates are labeled as prophylactic agents and preferred as the first line prophylactic medications [27,28]. The opportunity of home treatment provides an important support for the preference of this medication. Whereas, in Turkey, pdC1-INH concentrates are not licensed for prophylaxis and used as off-label only in selected patients when needed. Furthermore, home treatment is also not authorized. For these reasons and the inclusion of only adult patients in the study, the frequency of long-term prophylaxis with danazol was higher in our cohort. Its lower cost and easy usage compared to C1-INH constitute its advantages [25]. The adverse events due to danazol are dose-dependent and the dosages below 200 mg/day are usually safe. In our patients, danazol was used effectively without any significant side effects. Therefore, in carefully selected patients, attenuated androgens could be used for long-term prophylaxis.

As another important finding of the study, most of the patients under long-term prophylaxis were adherent (67.5%). The main reason for adherence was the efficiency of the drug in preventing serious attacks without causing an important side effect. The most common cause of nonadherence was fear of side effects (20.9% of nonadherers). Interestingly, patients with no family history, those having few relatives with HAE

and those having had no excitus due to HAE in their families were more adherent to the prophylactic treatment.

In our series, the delay until HAE diagnosis was approximately 18 years, a finding from another major city in Turkey in accordance with those of Ucar et al. who reported a diagnostic delay of approximately 17 years [29]. However, the first hereditary angioedema study conducted in Turkey reported the mean delay time as approximately 26 years [30]. This change is a satisfactory one, suggesting that the awareness of the disease has increased over the years in our country, yet it is inadequate. The delay in diagnosis widely changes depending on the individual countries as well. Zanichelli et al., reported median delay of 8.5 years in Europe [31]. Sixteen years in China and an American study reported 21 years delay in diagnosis recently [21,23]. These results again underline the lack of awareness of HAE around the world. Therefore, more efforts and perhaps new ideas are needed to increase the knowledge of the disease among both physicians and the public.

The demographic features of our patients were in accordance with previous HAE cohorts from Turkey and other countries in most aspects. In the current study, the majority of the patients were female and the onset age of symptoms was approximately 12 years. These features were in line with previous studies [8,19,30,33]. The frequency of type II HAE in our study (6.7%) was lower than some of the previous studies in which the frequencies were 15% and 20.3% [32,34] However, newer studies published similar prevalence of type II HAE ranging from 4.9% to 6.2% [5,6,20,35].

In conclusion, most of the HAE patients in our group were effectively under long-term prophylaxis with danazol and the compliance with the long-term prophylaxis was high. The most common cause of not using danazol regularly was the fear of side effects. Furthermore, the patients who lost their relatives due to HAE attacks and had more ill relatives in the family were less adherent to the long term prophylactic treatment.

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Ameliorating effects of exercise on disrupted epididymal sperm parameters in high fat diet-induced obese rats

Yüksek yağlı diyetle indüklenmiş obez sıçanların bozulmuş epididimal sperm parametreleri üzerine egzersizin iyileştirici etkisi

Merve AÇIKEL ELMAS, Serap ARBAK, Feriha ERCAN

ABSTRACT

Objective: Obesity causes male infertility problems and affects the sperm quality. Recent studies have shown that exercise has positive effects on male fertility. The present study aimed to show the effects of swimming exercise on the epididymal sperm number, motility and morphology in high fat diet (HFD)-induced obese rats.

Materials and Methods: Four experimental groups (n=8 in each group) were formed. Standard (STD) and STD+Exercise (STD+EXC) groups were fed with standard rat diet (6% of calories as fat); HFD and HFD+Exercise (HFD+EXC) groups were fed with high fat diet (45% of calories as fat) for 18 weeks. The rats in STD+EXC and HFD+EXC groups were trained by swimming sessions (1 h per day for 5 days a week) during the last 6 weeks of the experiment. The left caudal epididymis was prepared to evaluate the number, motility and morphology of the spermatozoa. The right epididymal samples were processed for histological evaluation.

Results: Normospermic parameters were seen in STD and STD+EXC groups. Sperm number and motility decreased and spermatozoa with abnormal morphology increased significantly in HFD group when compared with STD group. A large number of spermatozoa in the epididymal duct lumen and regular morphology of the fibromuscular connective tissue were observed in STD and STD+EXC groups. Most of the epididymal ducts consisted of decreased amount of spermatozoal accumulation in the HFD group. Degenerated pseudostratified columnar epithelium with vacuole formation were additional findings in this group. On the other hand, swimming exercise had an enhancement effect on sperm parameters with prominent spermatozoal accumulation in the ducts of epididymis in HFD induced obese rats.

Conclusion: This study shows that HFD-induced obesity decreased the number and motility of spermatozoa, increased

abnormal spermatozoa and caused disrupted epididymal morphology. We hypothesize that exercise enhanced HFD-induced spermatogenic and epididymal damages by the regulation of scrotal heat and possible inhibition of oxidative damage in the epididymis.

Keywords: High fat diet, Exercise, Sperm parameters

ÖZ

Amaç: Obezite erkek infertilite problemlerine neden olur ve sperm kalitesini etkiler. Egzersizin erkek fertilitesi üzerine olumlu etkileri olduğu çeşitli çalışmalar ile gösterilmiştir. Bu çalışmanın amacı, yüksek yağlı diyetle (YYD) indüklenmiş obez sıçanlarda yüzme egzersizinin epididimal sperm sayısı, motilitesi ve epididimis morfolojisi üzerine etkilerini göstermektir.

Gereçler ve Yöntemler: Bu çalışmada dört deney grubu (her grupta n=8) oluşturuldu. Standart (STD) ve STD+Egzersiz (STD+EGZ) gruplarındaki sıçanlar standart sıçan diyeti ile (kalisinde %6 yağ içeren); YYD ve YYD+Egzersiz (YYD+EGZ) grubundaki sıçanlar da yüksek yağlı diyet ile (kalisinde %45 yağ içeren) 18 hafta beslendiler. STD+EGZ ve YYD+EGZ grubundaki sıçanlara deneyin son 6 haftasında haftada 5 gün, günde 1 saat yüzme egzersizi yaptırıldı. Deney sonunda sol kaudal epididimis sperm sayısı, motilitesi ve morfolojisinin incelenmesi için hazırlandı. Sağ epididimis dokusu da histolojik inceleme için hazırlandı.

Bulgular: STD ve STD+EGZ gruplarında normospermik parametreler gözlemlendi. STD grubu ile kıyaslandığında YYD grubunda sperm sayısı ve motilitesinin anlamlı olarak azaldığı ve morfolojik anomaliye sahip sperm sayısının arttığı gözlemlendi. STD ve STD+EGZ gruplarında epididimal kanal spermatozoa ile doluydu ve fibromusküler bağ dokusu düzenli morfolojide gözlemlendi. YYD grubunda epididimal kanalların çoğu azalmış sayıda spermatozoa kümesinden oluşuyordu. Ayrıca, epididimisin stereosilyalı yalancı çok katlı silindirik epitelinde yer yer vakuol oluşumları gözlemlendi. Egzersizin ise YYD ile indüklenmiş obez sıçanlarda sperm parametrelerini ve epididimis kanalındaki sperm kümesini arttırdığı gözlemlendi.

Sonuç: Bu çalışma, obezitenin sperm sayısını ve motilitesini azalttığını, anomalili sperm sayısını arttırdığını ve epididimis morfolojisini bozduğunu göstermektedir. Egzersizin ise, skrotal ıstıyı dengeleyerek ve epididimisteki olası oksidan hasarı engelleyerek YYD ile indüklenmiş spermatogenik ve epididimal hasarı iyileştirdiği düşünülmektedir.

Anahtar kelimeler: Yüksek yağlı diyet, Egzersiz, Sperm parametreleri

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Introduction

Obesity is an important health problem that is defined as having a body mass index (BMI) greater than 30 kg/m², causing type 2 diabetes, cardiovascular diseases, endocrine and respiratory disorders, immunodeficiency, various types of cancer, psychological problems and infertility in both sexes [1]. Even only paternally-induced obesity leads couples to consult assisted reproductive techniques. Recent studies showed that probable effects of paternal obesity in the formation of newborns were prone to chronic diseases such as obesity and diabetes [2,3].

Obesity changes sperm morphology, motility and function, causing deterioration of testis structure [4]. In recent years, it has been shown that obesity reduces fertility and affects embryonic health [3,5]. According to studies in obese animal models, high fat diet (HFD) had a decreasing effect on sperm capacitation and sperm binding ability when compared to control diet [4,6]. Furthermore, feeding with HFD caused impaired morphology of sperms and decreased levels of testosterone and sperm motility [2,4,6,7].

The spermatogenesis process is very sensitive to temperature. The optimal temperature is 34-35 °C in humans [4,8,9]. Increased testicular temperature leads to a decrease in sperm motility and an increase in sperm DNA damage [10,11]. Obesity increases scrotal temperature thus changes sperm production by increasing gonadal temperature [12]. Epididymal cells produce different proteins, glycoproteins, glycolipids and phospholipids which are released in the lumen, necessary for maturation and survival of sperms [13]. In obese males, a large amount of fat deposited in the scrotum may be a relevant cause for the formation of oxidative stress [14] and alters epididymal morphology and function. This situation can also cause the alteration of sperm parameters which are stored in the epididymal lumen.

Diet and exercise can prevent or even reverse the effects of obesity-induced damage on sperm function [4]. Experimental studies showed that diet and exercise ameliorated sperm parameters such as motility, morphology, and sperm DNA damage and increased both fertilization and development of blastocyst [2,15-17]. The aim of this study is to investigate the effects of swimming exercise both on epididymal morphology and epididymal sperm parameters such as sperm number, motility and morphology in HFD – induced obese rats.

Material and Methods

Animals

Sprague Dawley albino male rats (7 weeks old, 250-300 gr) taken from the Experimental Animal Implementation and Research Centre of Acibadem University were used in this study. The experimental study was approved by Acibadem University, Animal Care and Ethical Committee for Experimental Animals (2018-36).

Experimental Design

The rats were maintained at 22 ± 2 °C during the experimental period in a laboratory environment with a standard light/dark (12/12 h) cycle. Four experimental groups (n = 8 in each group) were formed for the study. Standard (STD) and STD+Exercise (STD+EXC) groups were fed with standard rat diet (6% of calories as fat). HFD and HFD+Exercise (HFD+EXC) groups were fed with high fat diet (45% of calories as fat) for 18 weeks [18,19]. The animals in STD+EXC and HFD+EXC groups were trained by daily swimming sessions for 1 h per day for 5 days/week in the last 6 weeks of the experimental period. At the end of the experimental procedure, animals were fasted for 6 h, weighted and then anesthetized by intraperitoneal injection of ketamine (0.9 cc/kg) and xylazine (0.7 cc/kg). Epididymis of left testis was removed for sperm analysis, and the epididymal samples of the right testis were obtained for histological analyses.

Histological Preparation

Sperm counting, motility rate and morphological evaluation

Left caudal epididymis were dissected and cut into the small pieces in all groups, then epididymal samples were placed in 5 ml Earle's Balanced Salts Solution (Sigma, USA). Following centrifugation, supernatant was removed. Routine density gradient method was applied for sperm evaluation. Following the removal of the supernatant, the pellet was diluted with 2 ml sperm washing medium (SAGE, UK) and centrifuged at 2000 rpm for 10 min. Then, supernatant was separated and pellet was diluted with 0.3 ml fertilization medium (SAGE, UK). Sperm counting and motility rate were analyzed from one drop of pellet sample and examined by using Macler Counting Chamber (Sefi Medical Instruments, Haifa,

Israel) at photomicroscope. Smear samples were fixed and dehydrated with 96% ethanol and stained with Diff-Quick kit (Medion Diagnostics, Grafelfing, Germany) for the morphological evaluation. One hundred spermatozoa were evaluated for head, neck and tail morphology of the spermatozoa under 100x immersion oil objective of the photomicroscope.

Light Microscopical Preparation

Right caudal epididymal samples were fixed for 72 hours with 10% neutral buffered formalin solution. After fixation, tissues were dehydrated through ascending alcohol series (70%, 90%, 96%, 100%) and cleared with xylene. Then, tissue samples were embedded in paraffin. Sections of approximately 5 µm of thickness were stained with hematoxylin and eosin (H&E).

All of the histological slides were examined and photographed with a digital camera (Olympus C-5060, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan).

Statistical Analysis

Data were analysed by using one-way analysis of variance (ANOVA). Differences between groups were determined with Tukey's multiple comparisons test. Significance of differences was taken at the level of $P < 0.05$. Calculations were done using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Results

The total body weight measurements revealed that rats of HFD and HFD+EXC groups (315.8 ± 12.73 , 319.6 ± 10.83 respectively) were heavier than the rats of STD and STD+EXC group (303.6 ± 9.48 , 306.0 ± 9.66 respectively). Compared with the STD group, a significant increase in rat weight was observed in the HFD ($P < 0.05$) and HFD+EXC ($P < 0.01$) groups.

The number of spermatozoa in HFD ($P < 0.001$) and HFD+EXC ($P < 0.01$) groups were significantly reduced compared to the STD group. Moreover, the number of spermatozoa were increased in the HFD+EXC group ($P < 0.01$) compared to the HFD group (Figure 1A).

The progressive motile spermatozoa were significantly reduced in the HFD group ($P < 0.001$) compared to the STD group, while progressive spermatozoa were significantly increased in the HFD+EXC group ($P < 0.001$) compared to the HFD group (Figure 1B).

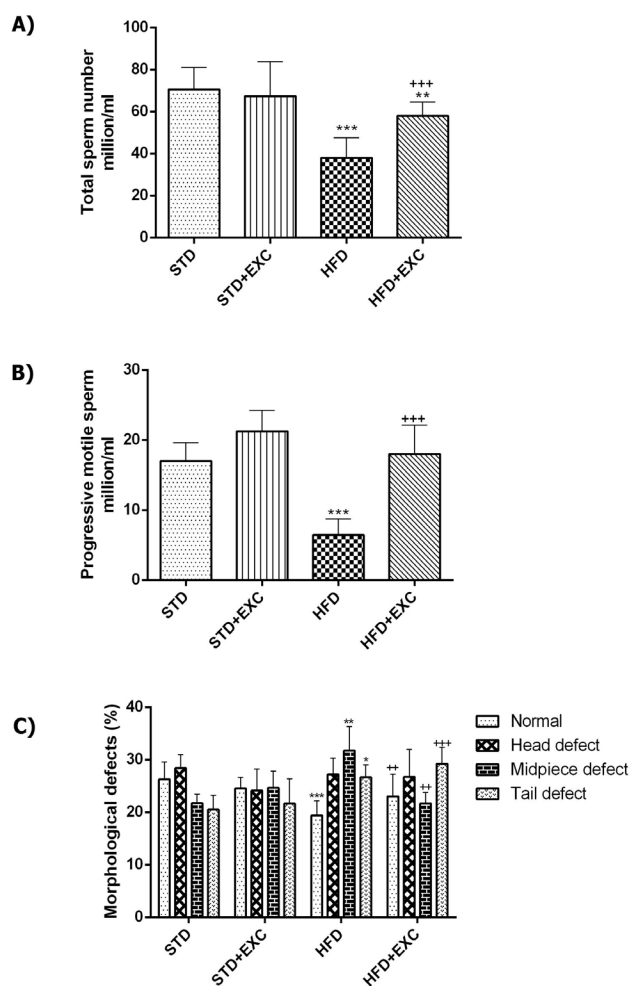


Figure 1. Evaluation of sperm count (A), sperm motility (B) and morphological defects (C), in the experimental groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to STD group; ** $P < 0.01$, *** $P < 0.001$ compared to HFD group.

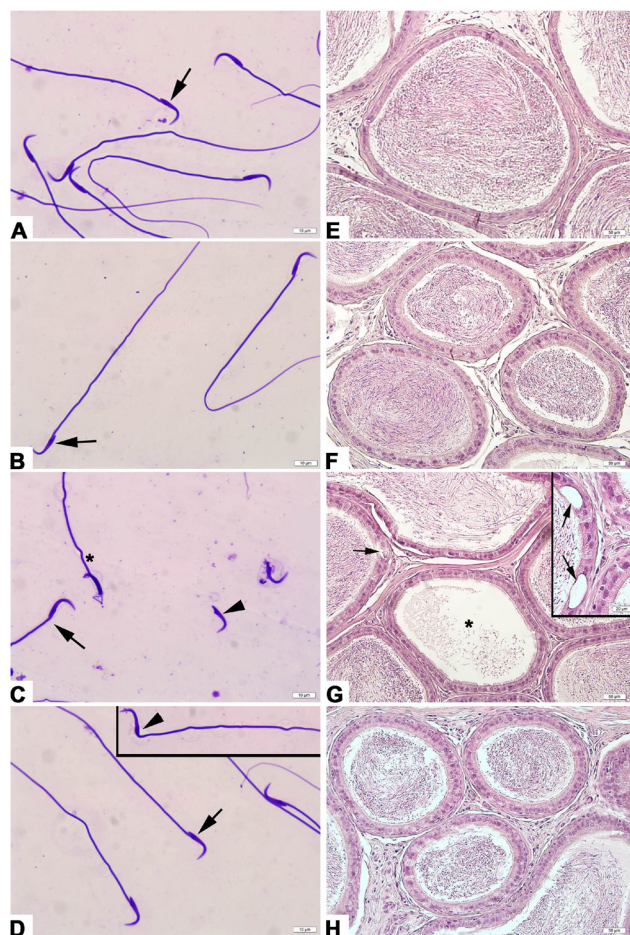


Figure 2. Representative light micrographs of experimental groups. Normal spermatozoa (arrow) and abnormal spermatozoa with mix (*) and neck (arrowhead) defects are seen in STD (A), STD+EXC (B), HFD (C) and HFD+EXC (D) groups. Regular epididymal duct morphology with accumulation of spermatozoa in luminal region of STD (E) and STD+ EXC (F) groups; decrease of sperm accumulation (*), epithelial degeneration with large vacuoles (arrow) in HFD group (G); quite regular epididymal ducts with sperm accumulation in HFD+EXC group (H) are seen in epididymis. A-D: Diff-Quick staining, E-H: H&E staining. Scale bar: A – D: 10 μ m; E-H: 50 μ m, inset: 20 μ m.

The spermatozoa with normal morphology and abnormal spermatozoa presenting head, midpiece and tail defects were observed in STD and STD+EXC groups. However, normal spermatozoa ($P < 0.001$) were significantly decreased and abnormal spermatozoa with midpiece ($P < 0.01$) and tail ($P < 0.05$) defects were significantly increased in the HFD group compared to STD group. Additionally, normal spermatozoa ($P < 0.01$) were significantly increased and abnormal spermatozoa with midpiece ($P < 0.01$) and tail ($P < 0.01$) defects were significantly decreased in the HFD+EXC group compared to the HFD group (Figure 1C and 2A-D).

Normal epididymal duct morphology, all together with luminal area, large numbers of spermatozoal accumulation were noticed in STD and STD+EXC groups. Decreased level of spermatozoal accumulation, immature spermatozoa in the luminal area, degeneration of stereociliated pseudostratified columnar epithelium with large vacuoles were observed in the HFD group. These morphological alterations were ameliorated in the HFD+EXC group (Figure 2 E-H).

Discussion

The present study reveals that HFD-induced obesity decreases the number, and motility of spermatozoa, and increases the incidence of degenerated spermatozoa. Furthermore, HFD disrupts the epididymal duct morphology. Moderate swimming exercise has been found to ameliorate HFD-induced sperm parameters and epididymal damages.

Body mass index is one of the important factors affecting fertility. As, BMI increases, the rate of infertility in men increases 3-fold [20]. Individuals with a BMI greater than 25 kg/m² have a lower total sperm count than those of normal weight, and the measured volume of ejaculate is decreased steadily with an increase in BMI [21].

Obesity results in harmful effects on the sperm parameters in males [15,16]. Similar adverse effects are also shown in rodent obesity models [22,23]. Obesity changes the environment crucial for spermatogenesis in testis and has an adverse effect on the sperm maturation in epididymis. Obesity impairs the physical and molecular structure of sperm during spermatogenesis and has an adverse effect on sperm maturation in epididymis. As a result, decreased sperm quality is associated with an increased risk in male infertility [24].

Hyperinsulinemia and hyperglycaemia, seen in obesity models of rats [2,7,25], change the number of sperm, impair sperm quality and cause decrease in fertility [26,27]. In addition, low testosterone level may cause clinical metabolic syndrome formation and so obesity may be a symptom of low testosterone level, although not directly causes low sperm count [28-30].

Obesity leads to an increase in adipocyte number/size and causes both physical and hormonal changes [24]. Physical changes cause an increase in scrotal temperature [12], On the other hand, hormonal changes might induce decrease of testosterone level [6]. These changes result in

oligozoospermia and azoospermia [31], a decrease in semen volume and contribute to obesity-related male infertility [32].

Diet and/or exercise interventions enhance basic sperm parameters such as motility and morphology in obese males. Limited number of studies showed the reversibility of the harmful effects of obesity [4]. Exercise increases sperm motility and morphology, reduces sperm DNA and oxidative damages [4]. Our study showed the ameliorating effects of exercise on sperm count, motility and morphology in HFD induced obese rats. This might be due to inhibition of oxidative and DNA damages in epididymal spermatozoa. Studies on obese mice have shown that exercise enhanced impaired sperm physiology [4]. An experimental study showed that FSH, LH, testosterone levels and semen quality increased in physically active individuals when compared to sedentary people [33]. Yet, in another study it has been shown that the semen parameters deteriorate in long-term heavy cycling people [34]. Although, moderate exercise increases testosterone/estrogen ratio and sperm quality, high-intensity exercise might have a negative or nonsignificant effect. Moderate swimming exercise enhanced sperm parameters in HFD-induced obese rats in this study. It can be concluded that the effects of exercise on the reproductive function of obese male rats might be related to the duration and intensity of exercise.

Testicular spermatozoa have no progressive motility and cannot fertilize oocytes, yet they acquire fertilization ability when they reach epididymis [13]. Therefore, epididymal tissue morphology and function are important for maturation of spermatozoa. For this reason, morphological and functional changes in the epididymis of obese animals may also change spermatozoal maturation. A large amount of adipose tissue accumulated in obese rats, probably causes oxidative stress in the epididymis [13]. In this study, it was observed that, epididymal ducts had degenerated epididymal epithelium with decreased amount of luminal sperm accumulation and increased immature spermatozoa in HFD-induced obese rats. Also, it was observed that sperm motility was reduced in this group. This data could be related with the altered epididymal secretion which has a role for acquiring sperm motility. Swimming exercise enhanced sperm motility in HFD-induced obese rats.

In conclusion, HFD-induced obesity in rats reduced the sperm number and progressive motility, increased the number of abnormal spermatozoa. Additionally, epididymal morphology was disrupted in this group which has a role for the maturation of spermatozoa. Swimming exercise ameliorated sperm parameters and epididymal morphology

by the regulation of scrotal heat and possible inhibition of oxidative damage in the epididymis.

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Effects of cholinergic compounds and TNF-alpha on human erythroleukemia K562 cell proliferation and caspase expression

Kolinerjik bileşiklerin ve TNF-alfanın insan eritrolösemi K562 hücre çoğalmasına ve kaspaz ekspresyonu üzerine etkileri

Zehra KANLI, Banu AYDIN, Hulya CABADAK

ABSTRACT

Objective: The purpose of this study was to investigate if stimulating auto-paracrine muscarinic receptor signalling pathway could change human erythroleukemia K562 cell proliferation and caspase 3, 8 and 9 expression levels. To better understand the role of muscarinic receptors in cell signalling mechanism, we investigated the effects of several compounds on human erythroleukemia K562 cell proliferation and caspase 3, 8 and 9 expression. These compounds were M₃ muscarinic receptor agonist, pilocarpine, pro-inflammatory cytokine, tumor necrosis factor (TNF)-alpha, and the wortmannin which is a phosphoinositide 3-kinase inhibitor.

Materials and Methods: Cell proliferation and cell viability were evaluated by the trypan blue exclusion test and 5-Bromo-2-deoxy-uridine (BrdU) Labelling and Detection Kits. Caspase 3, 8 and 9 expression levels were determined by immunoblot analysis.

Results: Both pilocarpine and TNF-alpha caused a small increase in human erythroleukemia K562 cell proliferation. However, when all the compounds were treated together, proliferation of human erythroleukemia K562 cells increased significantly when compared to untreated control cells. TNF-alpha and wortmannin treatment increased caspase 3 and caspase 8 expression patterns significantly in human erythroleukemia K562 cells. TNF-alpha and wortmannin treatment increased caspase 9 expression level (P>0.05) but it was not significant.

Conclusion: These findings partly demonstrated that M₃ muscarinic receptor mediated an increase in K562 cell proliferation. Pilocarpine prevented TNF-alpha and wortmannin induced caspase 3 and 8 expression and indirectly showed apoptosis in human erythroleukemia K562 cells.

Keywords: M₃ muscarinic receptors, Cytokine, Pilocarpine, Caspases, Erythroleukemia K562 cells

ÖZ

Amaç: Bu çalışmanın amacı, otoparakrin M₃ muskarinik reseptör sinyal yolağının uyarılmasının, insan eritrolösemi K562 hücrelerinin çoğalmasında ve kaspaz 3, 8 ve 9 ekspresyon seviyeleri üzerinde etkisi olup olmadığını araştırmaktır. Hücre sinyal ileti mekanizmasında muskarinik reseptörlerin rolünü daha iyi anlamak üzere, çeşitli bileşiklerin insan eritrolösemi K562 hücre proliferasyonu ve kaspaz 3, 8 ve 9 ekspresyon seviyeleri üzerindeki etkilerini araştırdık. Bu bileşikler, M₃ muskarinik reseptör agonisti, pilokarpin, pro-enflamatuvar sitokin, tümör nekroz faktör (TNF)-alfa ve fosfoinositid 3-kinaz inhibitörü wortmanindir.

Gereçler ve Yöntemler: Hücre çoğalması ve hücre canlılığı, tripan mavisi testi ve 5-Bromo-2-deoxy-uridine (BrdU) İşaretleme ve Belirleme kitleri ile değerlendirildi. Kaspaz 3, 8 ve 9 ekspresyon seviyeleri immunoblot analizi ile belirlendi.

Bulgular: Hem pilokarpin, hem de TNF-alfa, insan eritrolösemi K562 hücre çoğalmasında çok az artışa neden oldu. Ancak, tüm bileşikler birlikte muamele edildiğinde, insan eritrolösemi K562 hücrelerinin çoğalması, muamele edilmeyen kontrol hücrelerine göre anlamlı olarak arttı. TNF-alfa ve wortmanin ile muamele edilen K562 hücrelerinde kaspaz 3 ve kaspaz 8 ekspresyonu seviyelerinde anlamlı değişim belirlendi. TNF-alfa ve wortmannin muamelesi kaspaz 9 ekspresyon seviyesini arttırdı (P> 0.05) ancak anlamlı değildi.

Sonuç: Bu bulgular, kısmen M₃ muskarinik reseptör aracılı K562 hücre çoğalmasında artış olduğunu göstermektedir. Pilokarpin, insan eritrolösemi K562 hücrelerinde, TNF-alfa ve wortmanin ile uyarılan kaspaz 3 ve 8 ekspresyonunu önledi ve dolaylı olarak apoptozu gösterdi.

Anahtar kelimeler: M₃ muskarinik reseptörler, Sitokin, Pilokarpin, Kaspaz, Eritrolösemi K562 hücreleri

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Introduction

Chronic myelogenous leukemia (CML) which accounts for 20% of leukemia cases (annually 1-1.5/100.000 within a wide range of age width), is a malignant, clonal hematopoietic stem cell disorder [1]. Human erythroleukemia K562 cells derived from a 53-year-old female patient with CML in blast

crisis behaved as pluripotent hematopoietic stem cells. Thus, they can serve as a model system to study hematopoietic cell growth and differentiation [2]. Muscarinic receptors are G protein coupled receptors (GPCR). The M_1 , M_3 and M_5 muscarinic receptor subtypes (mAChRs) are coupled to $G\alpha_q$ that activates phospholipase C (PLC) enzyme which breaks down phosphatidylinositol 4, 5-bisphosphate (PI) into diacylglycerol (DAG) and inositol triphosphate (IP_3). The M_2 and M_4 mAChRs are coupled to $G\alpha_i/G\alpha_o$ and inhibit adenylate cyclase leading to a decrease in the formation of intracellular cyclic adenosine monophosphate (cAMP) [3-5]. Different cancer types are expressed by M_3 muscarinic receptor subtype (mAChR). The M_3 mAChR induces more than one signalling pathway in different types of cancer cells. Muscarinic receptor signalling pathways are related to cellular growth, survival, inflammation, angiogenesis, invasion, migration, pro-survival and anti-apoptosis in cancer cells. The stimulation of M_3 muscarinic receptors has been linked to the phosphoinositide three kinase (PI3K)/Akt pathway, which plays a role in the growth and proliferation of astrocytoma cells [6]. Muscarinic receptors are relatively abundant in the central nervous system and peripheral parasympathetic nervous system [3]. Muscarinic receptors also mediate some cellular events in hematopoietic cells and other non-neuronal cell types [4]. Recent experiments showed that non-neuronal cells and tissues express a mixture of muscarinic receptor transcripts. Muscarinic receptor subtypes have different functions [5]. In our previous studies we showed that M_2 , M_3 and M_4 subtypes of mAChR were expressed in human erythroleukemia K562 cell line and muscarinic activation led to erythroleukemia cell proliferation dependent on the presence of fetal bovine serum (FBS) [7-9]. The treatment of serum-deprived human erythroleukemia K562 cells with carbachol (CCh), a cholinergic agonist, led to a significant increase in DNA synthesis. CCh, also could produce a decrease in the DNA synthesis in human erythroleukemia K562 cells supplemented with 1% or 10% FBS after starvation [9]. The results of our previous studies also showed that nitric oxide (NO) release increased through M_3 receptors in the human erythroleukemia K562 cells [7].

Programmed cell death or apoptosis involves the activation of a series of cysteine aspartyl-specific proteases (caspases) related pathway and cell morphology changes. Caspase activation mechanisms are important in the apoptotic signalling pathway. During apoptosis two apoptotic pathways have been determined. These are cell-surface death receptor-dependent extrinsic pathway that activates caspase 8 and 10 and the other, the mitochondria-dependent intrinsic pathway that activates caspase 9. Both

extrinsic and intrinsic pathways lead to changes in the caspase 3, 6 and 7 expression levels [10-11].

Tumor necrosis factor (TNF)-alpha has several roles in biological responses like stress response, cell proliferation, differentiation, apoptosis and inflammation [12]. TNF-alpha, is produced by the activated macrophages and by different types of cells. TNF-alpha can induce both pro- and anti-apoptotic signalling pathways. TNF-alpha binds to R1 and R2 subtypes of the TNF receptors. These receptors activate caspase 8 related pathway [13]. Previous studies showed that, TNF-alpha can trigger not only the cell death, but also cell survival pathway as well [14]. Wortmannin negatively regulates the PI3K/Akt pathway.

The aim of the present work was to investigate the role of M_3 muscarinic receptor agonist pilocarpine on proliferation and caspase 3, 8 and 9 expression levels in K562 cells in a medium supplemented with 1% FBS after starvation. K562 cells were stimulated with pilocarpine in the presence or absence of other compounds in a medium supplemented with 1% FBS after starvation. We further investigated the effects of pro-inflammatory cytokine, TNF-alpha, the PI3-kinase inhibitor and wortmannin on cell proliferation in K562 cells. It remains unclear whether muscarinic M_3 stimulation contributes to the effects produced against the pro-inflammatory cytokines like TNF-alpha and wortmannin.

Material and Methods

Cell line and antibodies were used in the study. K562 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). 5-Bromo-2-deoxy-uridine (BrdU) Labelling and Detection Kits were supplied from Roche Company (Mannheim, Germany). The antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Secondary antibodies were purchased from Sigma (StLouis, MO, USA). Pilocarpine, TNF-alpha and wortmannin were purchased from Sigma-Aldrich Company (St. Louis, MO). Pilocarpine was prepared as 100 mM stock in distilled water, filtered through 0.2 μ m filter, and stored at -80°C . TNF-alpha was prepared as a 1 ng/ml solution in dimethyl sulfoxide (DMSO) and wortmannin was prepared as a 1 μ M solution in DMSO.

Cell proliferation assay

Cells were seeded into cell culture dishes and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 2mM-L-glutamine, 1% heat-inactivated

FBS, 100 U/mL penicillin, 100 lg/mL streptomycin at 37 °C under a humidified condition of 95 % air and 5 % CO₂. Cell viability and proliferation were also evaluated by the trypan blue exclusion test and cell counter (TC-20 BioRad, Hercules, CA, USA). Live and dead cells were distinguished by trypan blue exclusion test. The cell proliferation assay was done by using a BrdU kit. BrdU kit protocol was described in our previous study [8]. 100 ml of passaged K562 cells (1×10⁴cells) were seeded into 96 well plates containing RPMI-1640 medium without FBS. After 24 h, these “starved cells” were placed into a medium containing 1% FBS. The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% heat-inactivated FBS, 100 U/mL penicillin, 100 lg/mL streptomycin, at 37 °C under a humidified condition of 95 % air and 5 % CO₂. One of the following was then added to TNF-alpha (1ng/ml), and /or wortmannin (1mM) and 30 min later pilocarpine (100 mM) was added and left for 24 h. K562 cells cultured in RPMI-1640 medium (Sigma, USA) containing 1% FBS in 5% CO₂ incubator at 37°C, constituted the control group.

Preparation of whole-cell lysates and immunoblot analysis

Cells were collected at 400 g and washed two times with phosphate buffered saline (PBS). The resulting pellets were resuspended and lyzed with 20 strokes in a hand dounce homogenizer in a buffer containing 20 mM Hepes-KOH, pH 8.0, 0.1 mM, ethylenediamine-tetraacetic acid (EDTA), 0.1mM phenylmethylsulfonyl fluoride, 10mg/mL leupeptin and 2 mg/mL aprotinin. The protein content of the whole lysates was determined by the method of Lowry et al. [15]. Immunoblot analyses were described in our previous studies with minor modification [9]. 100 mg of protein was used in immunoblots and blots incubated overnight at 4°C with antibodies against caspase 3, 8, 9 and b-actin in separate blots. Caspases 3, 8 and 9 were quantified versus b-actin on the blot. The apparent molecular weights of caspases 3, 8 and 9 were 11 kDa, 20 kDa, 46 kDa, respectively. The blots were analyzed by densitometry. Total amount of protein in each lane was normalized to the endogenous b-actin control. Densitometric analysis was carried out with the free edition of the Bio-Rad Molecular Analyst Software Program.

Statistical analysis

All figures show mean (SD) of at least six independent experiments. Statistically significant differences were determined by using the one-way analysis of variance

followed by Dunnett’s post-tests. All statistical tests were performed with the Prism program (Graphpad Software) and (P<0.05) was considered significant. Asteriks were used to describe value levels of statistical significance (*P < 0.01; **P<0.003; ***P<0.003).

Results

Cells were stimulated with 100 μM pilocarpine, 1ng/ml TNF-alpha, 1 μM wortmannin for 24 h, and then cell viability and BrdU assay were carried out. The roles of M₃ muscarinic receptor agonist, pilocarpine, TNF-alpha, cytokine and PI3-kinase inhibitor and wortmannin, on proliferation of K562 cells are seen in (Figure 1). As shown in Figure 1, treatment of K562 cells with TNF-alpha in the absence of wortmannin had a minor stimularity effect on BrdU incorporation (Figure 1), while treatment with (100 μM), TNF-alpha and wortmannin together had additive stimulatory effect on BrdU incorporation. When TNF-alpha and wortmannin were added in the absence of pilocarpine, the number of BrdU labeled cells increased to 19.59 ± 2.1% of the control. TNF-alpha, wortmannin and pilocarpine increased the number of BrdU-labeled cells to 22.30± 2.20 % of the control group. There was no significant difference between the group treated with pilocarpine and the control group in the medium supplemented with 1% FBS after starvation. The group treated with TNF-alpha + wortmannin on BrdU incorporation also increased cell proliferation but to a lesser degree when compared to the control group (P< 0.05). Another group of cells that was treated with TNF-alpha+ pilocarpine + wortmannin on BrdU incorporation also increased significantly compared to the control group (P<0.05). The roles of M₃ muscarinic receptor agonist, pilocarpine, TNF-alpha, and PI3-kinase inhibitor, wortmannin on caspase 8, 9 protein expression in K562 cells were shown in figures 2 and 3. Inhibition of PI3K/Akt by wortmannin influenced the expression of caspase 8 overtly (Figure 2A) and caspase 3 (Figure 3). However, inhibition of PI3K had no effect on the expression of caspase 9 (Figure 2B). The increased caspase 3 expression induced by TNF-alpha and wortmannin was significantly attenuated by the addition of pilocarpine (Figure 3). These results suggested that pilocarpine could protect K562 cells from TNF-alpha and wortmannin induced apoptotic signalling pathway. We observed that combination of PI3Kinase inhibitor, wortmannin and TNF-alpha increased expression of caspase 3, which is executioner or effector caspase and caspase 8, which is an initiator caspase (Figure 2A and Figure 3). Wortmannin specifically inhibited the phosphatidylinositol 3-kinase pathway, that could be used as a

promising apoptosis-based therapeutic agent with TNF-alpha in the treatment of leukemia. Flowchart about the mechanism involved in TNF-alpha and wortmannin mediated apoptosis in human erythroleukemia K562 cells was shown in Figure 4.

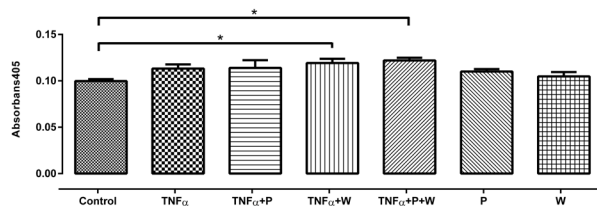


Figure 1. The effects of pilocarpine (100 mM) alone or in the presence of wortmannin (1μM), TNF-alpha (1ng/ml) on human erythroleukemia K562 cell proliferation. Cells (1×10⁴ cells/well) were disseminated in 96 well dishes in the absence of fetal bovine serum and cultured for 24 h and then 1% fetal bovine serum was added with or without pilocarpine, wortmannin and TNF-alpha. The cells were pre-treated with wortmannin 30 min before addition of TNF-alpha. BrdU was applied for the last 4 h. Each bar represents the mean ±SEM of four independent experiments. *P<0.05 between control versus TNF-alpha+wortmannin, TNF-alpha +pilocarpine +wortmannin

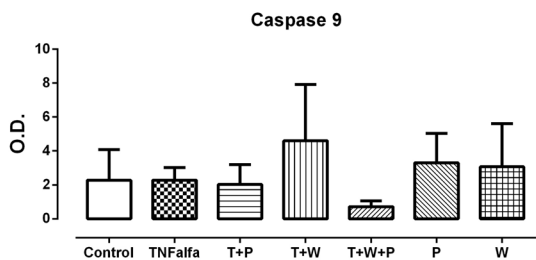
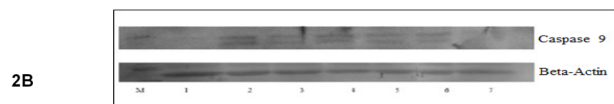
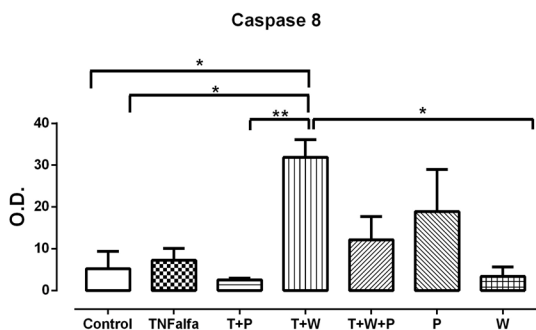
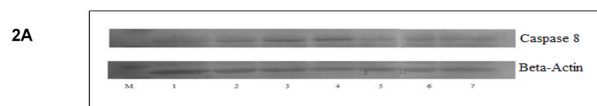


Figure 2. The effect of pilocarpine on the expression of caspase 8 and 9 proteins. K562 cells were treated with wortmannin (1

μM) for 30 min prior to exposure to TNF-alpha (1ng/ml) and / or pilocarpine. Western blot analysis for expression of caspase 8 and 9 was performed on whole lysates. (A) Apoptosis initiating protein levels of caspase 8 (20 kDa) and (B) caspase 9 (46 kDa) were detected by Western blotting. β-Actin was used to normalize the amount of protein loaded in each lane. Representative Western blot images are shown. Values are expressed as the mean ± SEM of four independent experiments. *P<0.003; **P<0.01.

O.D.: Optical Density, T: TNF-alpha, P: Pilocarpine, W: Wortmannin. 1-Control, 2-TNF-alpha, 3 - T+P (TNF-alpha + pilocarpine), 4-T+W (TNF-alpha+ wortmannin), 5 - T+W+P ((TNF-alpha+wortmannin + pilocarpine)

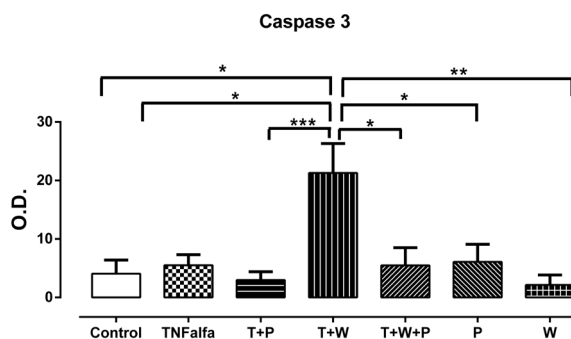
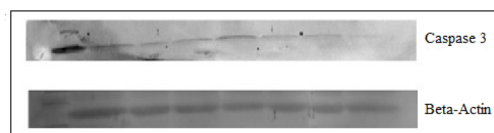


Figure 3. The effect of pilocarpine on the expression of caspase 3 protein. K562 cells were treated with wortmannin (1 μM) for 30 min prior to the exposure of TNF-alpha (1ng/ml) with or without pilocarpine (100 μM). Western blot analyses for expression of caspase 3 were performed on whole lysates. Apoptosis effector protein of caspase 3 (11 kDa) was detected by Western blotting. β-Actin was used to normalize the amount of protein loaded in each lane. Values are expressed as the mean± SEM of four independent experiments. *P<0.003; **P<0.01; ***P<0.003.

O.D.: Optical Density, T: TNF-alpha, P: Pilocarpine, W: Wortmannin. 1-Control, 2-TNF-alpha, 3 - T+P (TNF-alpha + pilocarpine), 4-T+W (TNF-alpha+ wortmannin), 5 - T+W+P ((TNF-alpha+wortmannin + pilocarpine)

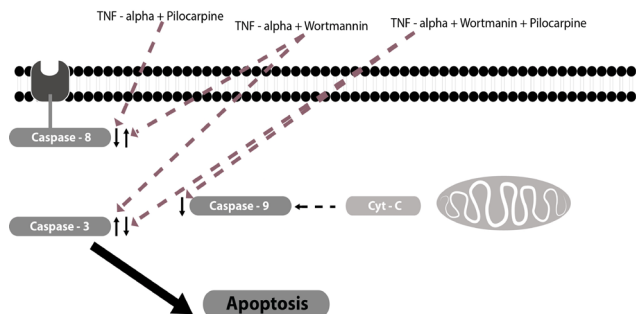


Figure 4. Schematic representation of the mechanism involved in TNF-alpha and wortmannin mediated expression of caspase 3, 8 and 9 during apoptosis of human erythroleukemia K562 cells.

These findings indicate that pilocarpine may act through M₃R signalling to prevent apoptosis and promote erythroleukemia cell proliferation, targeting that M₃R, PI3K and TNF-alpha might provide us a potential therapeutic strategy for leukemia treatment.

Discussion

In the present study, we have demonstrated the effects of TNF-alfa, pilocarpine and wortmannin on the cell proliferation and caspase 3, 8 and 9 expression levels in human erythroleukemia K562 cells. M₃ muscarinic receptor is expressed in different cancer types. These cancer types are the skin, colon, gastric, pancreatic, breast, ovarian, brain and lung [16]. TNF-alpha, produced by activated macrophages, is a cytokine that influences growth, differentiation and apoptosis in most cell types [17]. Different researchers suggested that TNF-alpha could also trigger cell survival pathway that induced NFkB pathway [18]. It was also documented that caspase 3 and 8 may have been the key regulators of the apoptotic response during tumorigenesis [19]. Gonza'lez-Flores and colleagues showed that TNF-alfa caused a time dependent increase in caspase 3, 8 and 9 activities in K562 cells [18]. Another group detected similar results in U937 cells [20] but in their study they used different FBS concentration in the cell proliferation medium. Sandra and colleagues showed that a low concentration of TNF-alpha in the presence of wortmannin or LY294002 induced apoptosis in a human head and neck squamous cell carcinoma (SAS) cell line. They suggested that the PI3K-NFkappaB pathway contributed to the TNF-alpha induced cell survival and that inhibition of this pathway accelerated apoptosis in the SAS cell line [21]. Various researchers showed that PI3K/Akt pathway could inhibit cell apoptosis [22, 23]. PI3K/Akt pathway is also involved in both proliferation and inhibition of apoptosis. It was also demonstrated that the cell proliferation could be evidently inhibited by wortmannin in a dose-dependent manner and wortmannin could arrest the cell cycle and induce cell apoptosis. It was also stated that, a lower concentration of wortmannin did not induce apoptosis [24]. Previously, we had shown that the PI3-kinase inhibitor wortmannin (1 µM) had an inhibitory effect on DNA synthesis. But, DNA synthesis was stimulated when wortmannin was added prior to CCh challenge. We also suggested that different signalling pathways participated in the muscarinic receptor mediated regulation of cell proliferation in one of our previous studies [9]. Exogenous muscarinic agonist,

pilocarpine stimulated cell growth in non-small cell lung cancer (NSCLC) A549 and PC9 cell lines but M₂ muscarinic antagonist, methoctramine inhibited tumor growth [25]. This study showed that pilocarpine caused a little increase in K562 cell proliferation when compared to the control group, while TNF-alpha and wortmannin had additive effects in increasing K562 cell proliferation in 24 h. TNF-alpha, pilocarpine and wortmannin together increased K562 cell proliferation when compared to the control group. The intracellular pathway compounds had biological effects on these cells. Our results led us to conclude that pilocarpine could protect cells from apoptosis. In the present study, when TNF-alpha, PI3K inhibitor and wortmannin were administered together, they induced apoptosis which involved pathways of receptor-mediated apoptosis. But, they did not affect the mitochondrial pathway in K562 cells. As shown, in this study TNF-alpha and wortmannin induced apoptosis via caspase 8 which initiated receptor-mediated apoptosis, but TNF-alpha and wortmannin did not affect caspase 9, mitochondrial cell death pathway in K562 cells, significantly.

Our study suggested that the treatment of human erythroleukemia K562 cells with TNF-alpha and wortmannin induced caspase 3 and 8 expression in the absence of pilocarpine. But, addition of pilocarpine decreased caspase 3 and 8 expression within 24 h in K562 cells. Our results implied an indirect contribution to the apoptotic pathway in human erythroleukemia cells, since we only detected caspase 3, 8 and 9 protein levels. Finally, we provided a flowchart about the mechanisms involved in TNF-alpha and wortmannin mediated apoptotic pathway proteins for the protective effects of pilocarpine in human erythroleukemia K562 cells. M₃ receptor-mediated suppression of TNF-alpha and wortmannin effects may be important protective mechanisms of apoptosis in the erythroleukemia cells.

In conclusion, our results suggested that TNF-alpha and wortmannin together stimulated caspase 3, 8 expression and finally increased apoptosis in human erythroleukemia cells.

Pilocarpine prevented TNF-alpha and wortmannin induced apoptotic protein and expression of caspase 3 and 8 in K562 cells, resulting in the inhibition of TNF-alpha and wortmannin mediated caspase expression. We hypothesize that this may protect human erythroleukemia cells from apoptosis. The suppressive effect of pilocarpine was further demonstrated by the decreased expression of caspase 3, which acted as an essential executor and expression of caspase 8, a biomarker of apoptosis in mammalian cells, which initiated

receptor-mediated apoptosis. Our findings may partly explain the mechanisms underlying the protective effect of M₃ muscarinic receptors in apoptosis. The involvement of M₃-muscarinic receptors in the protection of cell death induced by TNF-alpha and wortmannin was supported by the up-regulation of PI3K pathway.

In summary, pilocarpine via M₃ muscarinic receptor appeared to have an inhibitory function and could be regarded as an important mediator in TNF-alpha and wortmannin mediated erythroleukemia cell apoptosis.

Compliance with ethical standards: Ethical approval is not needed (Cell line culture). K562 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD).

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Investigation of the effectiveness of algan hemostatic agent in bleeding control using an experimental partial splenectomy model in rats

Deneyisel parsiyel splenektomi modelinde sıçanlarda algan hemostatik ajanın kanama kontrolünde etkinliğinin araştırılması

Ahmet MIDI, Husamettin EKICI, Ali KUMANDAS, Omercan DURMUS, Buse BODIC, Mehmet TIRYAKI, Mehmet Sabri BALIK, Erdem YESILADA

ABSTRACT

Objective: Algan hemostatic agent (AHA) is a plant-based hemostatic agent produced in Turkey. Although, there is a great improvement in the hemostatic technologies, more effective hemostatic products are required to be produced. The aim of this study was to demonstrate the efficacy of AHA in a partial splenectomy model in rats. In addition, in this model, postoperative abdominal adhesion was evaluated.

Materials and Methods: In this study 5-7 weeks old 64 rats were used. Rats were randomly divided into 8 groups, each consisting of eight rats (4 groups heparinized and 4 groups non-heparinized). Experimental splenectomy was performed and the gauze impregnated with saline was applied to the control group for the hemorrhage control, the gauze impregnated with liquid AHA, gel and powder form of AHA, was applied to the experimental groups.

Results: The time to reach complete homeostasis was significantly shorter in all AHA groups compared to the control

group. The powder and the gel forms of AHA stopped the bleeding in heparinized and non-heparinized groups in 1 second. The AHA fluid (sponge) form stopped the bleeding in the first application in the control group less than 10 seconds and the second time application was not necessary. The bleeding was able to be controlled in the heparinized control group (saline impregnated sponge) by 55 seconds and in the non-heparinized control group by 38 seconds.

Conclusion: This study showed that AHA is a highly effective hemostatic agent, which would be beneficial in controlling hemorrhage.

Keywords: Algan hemostatic agent, Hemostasis, Rat, Bleeding control, Splenectomy

ÖZ

Amaç: Algan hemostatik ajan (AHA), Türkiye’de üretilen bitki bazlı hemostatik bir maddedir. Hemostatik teknolojilerdeki büyük gelişmelere rağmen, kanamanın kontrolünde kullanılmak üzere daha etkili hemostatik ürünlerin üretilmesi gerekmektedir. Bu çalışmanın amacı, AHA’nın sıçanlarda parsiyel splenektomi modelindeki etkinliğini göstermektir. Ayrıca bu modelde batın yapışıklığı değerlendirilmiştir.

Gereçler ve Yöntemler: Bu çalışmada 5-7 haftalık 64 sıçan kullanıldı. Sıçanların her biri sekiz sıçandan oluşan 8 gruba ayrıldı (heparinize edilmiş 4 grup ve heparinize edilmemiş 4 grup). Deneyel splenektomi yapıldı ve kontrol grubuna hemoraji kontrolü için serum fizyolojik emdirilmiş gazlı bez uygulandı, deney gruplarına AHA sıvı emdirilmiş gazlı bez, jel ve toz formu uygulandı.

Bulgular: Komple hemostaza ulaşma süresi, tüm AHA gruplarında kontrol grubuna kıyasla anlamlı olarak daha kısa idi. AHA’nın toz ve jel formları heparinize ve heparinize olmayan gruplarda 1 saniyede kanamayı durdurdu. AHA sıvı (sünger) formu, kontrol grubunda ilk uygulamada tüm sıçanlarda kanamayı durdurdu (10 saniyenin altında). Kanama, heparinize edilmiş kontrol grubunda (serum fizyolojik emdirilmiş gazlı bez) ortalama 55 saniyede ve heparinize olmayan kontrol grubunda 38 saniyede kontrol edilebildi.

Sonuç: Bu çalışma, AHA’nın hemorajinin kontrol edilmesinde yararlı olabilecek oldukça etkili bir hemostatik ajan olduğunu göstermiştir.

Anahtar kelimeler: Algan hemostatik ajan, Hemostaz, Sıçan, Kanama kontrolü, Splenektomi.

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Introduction

The reason for many splenectomies nowadays is spleen bleeding caused as a result of elective spleen surgeries applied due to several medical reasons and especially trauma. The spleen is the second most frequently injured organ after abdominal traumas and missed splenic injury is the most frequent preventable cause of death in patients with trauma. For this reason, there are studies on partial spleen protection and in control of spleen hemorrhage following trauma [1-3]. In the early 1900s, the mortality rate for non-surgical treatment of splenic injuries was approximately 100%. For this reason, splenectomy was widely acknowledged treatment option in spleen injuries [4].

Today, for many patients with solid organ damage, laparotomy is not necessary by virtue of imaging techniques. Since, non-surgical treatments in solid organ injuries give better results comparing to the surgical treatments, non-surgical treatment options are highlighted. Besides, the risk of postoperative infection in splenectomy is elevated in spleen injuries, therefore the approaches protecting the spleen come to the forefront. There are many hemostatic agents available such as; bovine collagen, bovine thrombin, autologous plasma, fibrin glue [5-12], and it is necessary to decide which method to use according to the cost of the procedure, bleeding severity and personal experience. However, despite these products and major developments in medicine, an ideal product that can be used to control bleeding is not yet produced and more effective hemostatic products are necessary to be produced.

The algal hemostatic agent (AHA) is the herbal extract derived from the standardized blend of six different plants (Table I) [13,14]. To the best of our knowledge, it is the first and only patented product in the world, made solely of herbs, with no additives. (Patent application no: a2015 / 00018, application publication no. TR2015 0018 A2).

All biocompatibility tests such as sensitization, cytotoxicity and irritation and hemodynamic tests of the AHA had been performed, and the results supported its safety and efficacy as a hemostatic agent [13,14]. AHA can be easily formulated to be applied locally [13,14]. Further, it has low cost and does not require special storage conditions.

The aim of this study is to assess the hemostatic effect of the partial splenectomy model of the AHA. In addition, postoperative abdominal adhesion is evaluated.

Table I. Plants with algal hemostatic agent composition.

The name of the plant	English name	Used part
<i>Achillea millefolium</i>	Yarrow	Flower
<i>Juglans regia</i>	Walnut	Leaf
<i>Lycopodium clavatum</i>	Club moss	Whole plant
<i>Rubus caesius</i> , <i>R. fruticosus</i>	Blackberry	Leaf
<i>Viscum album</i>	European Mistletoe	Whole plant
<i>Vitis vinifera</i>	Vine	Leaf

Materials and Methods

This study was approved by the Institutional Animal Experiments Local Ethics Committee of Kırkkale University (number, 2018/10). All animal studies conformed to the animal experiment guidelines of the Committee for Humane Care. All animals received care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and “Guide for the Care and the Use of Laboratory Animals” prepared by the US National Academy of Sciences and published by the US National Institute of Health (NIH Publications, No:80-23). The experiment was carried out as described in the literature [1].

In the study, 64 rats which are 180-210 grams and 5-7 weeks old were used. Rats were fed as ad libitum and examined under standard laboratory conditions according to a 12-hour dark-light period. Rats were randomly divided into two groups each having 32 rats as; heparinized and non-heparinized groups. Subsequently, the experimental animals were randomly divided into 8 groups each having eight rats. The heparinized group was administered intraperitoneal 640 IU / kg heparin for 3 days once a day. The other group did not receive heparin. Group 1 (Heparinized control group), Group 2 (Heparinized AHA powder group), Group 3 (Heparinized AHA gel group), Group 4 (Heparinized liquid AHA-impregnated sponge group), Group 5 (Non-Heparinized control group), 6th group (Non-Heparinized AHA powder group), 7th group (Non-Heparinized AHA gel group), 8th group (Non-Heparinized AHA liquid impregnated sponge group).

Operation procedure

During the operations, all rats were treated according to the Guide for the Care and Use of Laboratory Animals. Procedures were performed under general anesthesia with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). All efforts were made to minimize animal suffering and the number of animals used.

The furs on the abdominal anterior wall of all rats were removed. After disinfection with povidone-iodine solution, 3 cm median incision was made. The spleen was located and in the lower pole of the spleen 1 cm, partial splenectomy was performed in the rats in all groups. The duration of bleeding was evaluated according to the protocol previously explained [12]. When splenectomy bleeding had started, the bleeding area was pressed with a sponge for 10 seconds, and then the sponge was removed. Liquid AHA-impregnated sponge, and the saline-impregnated sponge were placed in this area and a light pressure was applied to the area. Bleeding was checked 10 seconds after the start of the pressure. If bleeding stopped, it was recorded as 'bleeding stopped'. If not, the procedure was repeated until the bleeding was controlled with the same amount of material. In the AHA gel and AHA powder forms, the bleeding area was left unpressed and was left open after the application. Bleeding was checked. The time at which the bleeding stopped was noted as the time of the bleeding control (Figure 1).

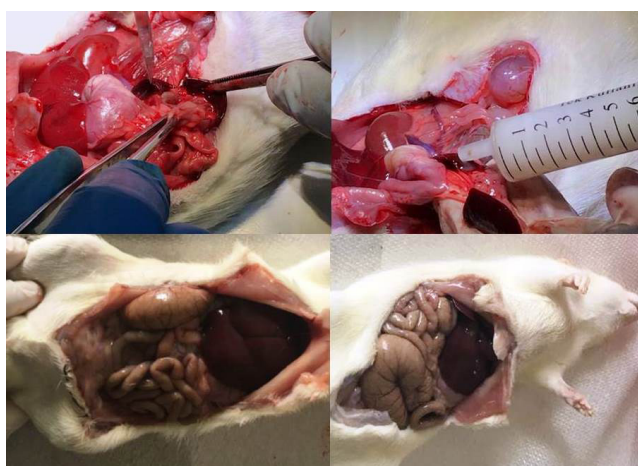


Figure 1. The images of the spleen cut from the center and the application of the AHA gel. On the postoperative 5th day, there is no adherence in the abdomen in the control and AHA liquid groups

On the 5th day of the laparotomy, the rats underwent surgical site disinfection on the anterior wall of the abdomen under anesthesia. The abdomen was opened and the intra-abdominal adhesiveness quantitatively evaluated according to the Bothin scale [12]. Hematoma and fluid collection were examined in the abdomen, if it existed. The rats were sacrificed by cutting inferior vena cava.

Statistical analyses

SPSS software version 22.0 (SPSS Inc., Chicago, IL) was used to analyze the data of this study. Weight, bleeding time and adherence scores were calculated and mean values were compared among the four groups using analysis of variance (ANOVA). When differences were found, any group difference was determined by Duncan's multiple range tests. The results were assessed at a 95% confidence interval and a suggestiveness level of $P < 0.05$.

Results

The shortest duration of bleeding was found in the AHA powder group. This was followed by the gel group and the liquid group. The duration of bleeding in the control group was significantly longer than in the experimental groups (Table II). There was no difference in terms of body weights between the groups. The powder and the gel forms of AHA stopped the bleeding in heparinized and non-heparinized groups in 1 second. The AHA fluid (sponge) form stopped the bleeding in the first time in control group less than 10 seconds and the second time application was not necessary.

The saline-impregnated sponge was able to control bleeding in the heparinized control group at the average of 55 seconds (min: 40-max: 70 sec) and in the non-heparinized

Table II. Mean bleeding time and the body weight distribution of the groups.

	Group 1 (HC)	Group 2 (HP)	Group 3 (HG)	Group 4 (HL)	Group 5 (NHC)	Group 6 (NHP)	Group 7 (NHG)	Group 8 (NHL)	P
Weight (gram)	190.6 ± 5.5	189.5 ± 7.3	183.6 ± 10.6	194.6 ± 5.7	175.6 ± 10.5	182.4 ± 7.5	187.9 ± 7.5	184.3 ± 9.5	$P > 0.05$
Average Bleeding time, second (Min-max)	55 (40-70 sec.) (Min-max)	1 sec.	1 sec.	< 10 sec. (at first control)	38 sec. (30-50 sec.) (Min-max)	1 sec.	1 sec.	< 10 sec. (at first control)	$P < 0.001$

HC: Heparinized control group, HP: Heparinized AHA powder group, HG: Heparinized AHA gel group, HL: Heparinized AHA liquid-impregnated sponge group, NHC: Non-Heparinized control group, NHP: Non-Heparinized AHA powder group, NHG: Non-Heparinized AHA gel group, NHL: Non-Heparinized AHA liquid-impregnated sponge. AHA: Algan Hemostatic Agent.

Table III. Assessment of the adhesion forms of the groups (%)

Adhesion Zone	Group 1 (HC)	Group 2 (HP)	Group 3 (HG)	Group 4 (HL)	Group 5 (NHC)	Group 6 (NHP)	Group 7 (NHG)	Group 8 (NHL)	P
Between omentum and target organ	6	7	6	6	6	7	7	6	>0.05
Omentum abdominal scar	0	0	0	0	0	0	0	0	
Between omentum and other sites	0	0	0	0	0	0	0	0	
From adnexa to target organ	0	0	0	0	0	0	0	0	
Adnexa abdominal scar	0	0	0	0	0	0	0	0	
From adnexa to other places	0	0	0	0	0	0	0	0	
Adhesive tape between any two organs	0	0	0	0	0	0	0	0	
Target organ abdominal scar	0	0	0	0	0	0	0	0	
Target organ abdominal wall	0	0	0	0	0	0	0	0	
Target organ intestine	2	1	1	2	2	1	0	1	
Target organ liver	0	0	0	0	0	0	0	0	
Adhesion in any other organ	0	0	0	0	0	0	0	0	
Total Adhesion Score	8	8	7	8	8	8	7	7	

HC: Heparinized control group, HP: Heparinized AHA powder group, HG: Heparinized AHA gel group, HS: Heparinized AHA liquid impregnated sponge group, NHC: Non-Heparinized control group, NHP: Non-Heparinized AHA powder group, NHG: Non-Heparinized AHA gel group, NHL: Non-Heparinized AHA liquid impregnated sponge. AHA: Algan Hemostatic Agent.

control group at 38 (min: 30-max: 50 sec) seconds (Table II).

There was no hematoma and fluid accumulation in the abdomen on the postoperative 5th day. The lowest adhesion score was 7 and the highest was 8. There was no difference in the adhesion scores between the control and treatment groups (Table III).

All of the applications were performed with the sterile AHA in a sterile environment. All rats in the treatment and control groups were alive on the 5th postoperative day. On the postoperative 5th day, when the abdomen was opened, no surgical infection was observed in neither control nor the AHA groups.

Discussion

All forms of the AHA have stopped bleeding in the first second in the partial splenectomy bleeding model used in this study and we have shown that they are potential candidates as an effective product in the use of hemostasis in splenic injuries or partial splenectomy operations. In heparinized and non-heparinized powder and gel groups, the bleeding was stopped within 1 second. The AHA was significantly different from the control groups in terms of bleeding control efficacy.

Several hemostatic agents have been used in the treatment of solid organ hemorrhages [15-17], and they work by different mechanisms. Tissue adhesives, cyanoacrylates are used in many clinical situations for the hemostatic purposes [18-20].

In one study in the literature, hemostatic efficacy was compared in ankaferd and fibrin glue in a rat model of partial splenectomy [21]. In this study, Fibrin Glue (Tisseel®) was able to stop bleeding for an average of 11 seconds and Ankaferd Blood Stopper for an average of 10 seconds. As we have shown in the current study, AHA managed to stop the bleeding in as fast as 1 second.

In another study, the hemostatic effect of calcium alginate in experimental splenic injury model was investigated. In that study, a spleen laceration model was established and the hemostatic effect of calcium alginate was evaluated. Calcium alginate has been shown to reduce the intraoperative bleeding after spleen injury. When compared to the 0.9% NaCl gauze and sham groups, inflammation, vascularization, and fibrosis have been found statistically higher in calcium alginate group. And the adhesion score has also been found higher in the calcium alginate group [22].

Nowadays, many products used for hemostasis have some difficulties during application such as compression. However, AHA does not require compression compared

to the other products and it has an easy-to-apply feature, as well as an advantage over other products in terms of hemostasis in a much shorter time.

All forms of the AHA and the results of the effectiveness in hemostasis differ greatly from the other available products. Experimental conditions may vary in terms of animal weight, the experience of the practitioner, technical differences, vessel variations, laboratory conditions, and other factors affecting this disparity and the other bleeding arrestors. Therefore, all products need to be compared in the same experiment protocol to evaluate their efficacies in bleeding control.

Prevention of peritoneal adhesions is important issues in surgery. There are many agents such as phospholipase inhibitors, dextran, corticosteroids, phospholipids, and methylene blue used to prevent postoperative abdominal adhesions [23-25].

According to some studies in the literature, some of the haemostatic agents have postoperative abdominal adhesion enhancing effect. Other studies show the opposite of these results [22, 26, 27]. Our study showed that AHA did not have a positive or negative effect on postoperative intra-abdominal adhesion formation.

According to the results of this study and together with the other studies in the literature, AHA is a highly effective hemostatic agent in the partial splenectomy hemorrhage model in the world, but the actual difference can only be demonstrated by comparative studies. The future studies are needed to further this study.

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Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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A retrospective analysis of efficacy of systemic therapy in metastatic thyroid cancer

Metastatik tiroid kanserinde sistemik tedavi etkinliğinin retrospektif olarak değerlendirilmesi

Atakan DEMİR

ABSTRACT

Objective: Thyroid cancer is the most common type of endocrine cancer. The current approaches in the systemic therapies of metastatic thyroid cancers are chemotherapy that is investigated in phase II clinical trials and tyrosine kinase inhibitors, investigated in phase III clinical trials. The aim of this study was to evaluate the efficacy of systemic therapies in metastatic thyroid cancer patients.

Materials and Methods: We investigated 57 patients retrospectively, diagnosed with thyroid cancer who were referred to Maslak Acibadem Hospital Medical Oncology Department between 2008-2015 and Umraniye Training and Research Hospital between 2016-2018. They had received systemic treatment due to the refractory profile to radioiodine therapy and metastatic thyroid cancer.

Results: Medical records of 57 patients with metastatic thyroid cancer, who were referred for systemic therapy were retrospectively analysed. 52% (n:30) of the cases were women and 48% (n:27) were men, and the mean age was 57.11 years. All patients was above the age of 18. Of the patients, 59.8% (n:35) had well differentiated thyroid cancer, 29.8% (n:17) had medullary thyroid cancer, 5.3% (n:3) had anaplastic thyroid cancer, 3.5% (n:2) had poorly differentiated thyroid cancer and 1.8% (n:1) had medullary-papillary synchronous cancer. When first line systemic therapy was evaluated for all 57 patients, progression free survival (PFS) was found 4.25 and 6.33 months for chemotherapy and sorafenib, respectively (P:0.035). All cases were evaluated retrospectively for second line systemic therapy and PFS was 4.1 and 7.77 months for chemotherapy and sorafenib, respectively (P<0.001).

Conclusion: Tyrosine kinase inhibitors are used in the treatment of radioactive iodine-refractory differentiated thyroid cancers and medullary thyroid cancers. The effect of lenvatinib, sorafenib and vandetanib on progression-free survival in thyroid cancers is found to be superior to systemic chemotherapy. It was concluded that sorafenib is a systemic treatment option which can be preferred in terms of efficacy and toxicity profile in radioactive iodine refractory well-differentiated thyroid cancer especially in our country.

Keywords: Thyroid, Cancer, Sorafenib

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ÖZ

Amaç: Tiroid kanserleri, endokrin kanserleri içerisinde en sık görülen kanserdir. Metastatik tiroid kanserlerinin sistemik tedavisinde güncel yaklaşımlar faz II çalışması olan kemoterapi ve faz III çalışması tirozin kinaz inhibitörleridir. Çalışmamızda, metastatik tiroid kanserli hastalarda sistemik tedavilerin etkinliğini belirlemeyi hedefledik.

Gereçler ve Yöntemler : Maslak Acibadem Hastanesi Tıbbi Onkoloji Kliniği'ne 2008 ile 2015 yılları arasında ve 2016 ile 2018 yılları arasında Sağlık Bakanlığı Sağlık Bilimleri Üniversitesi Umraniye Eğitim ve Araştırma Hastanesi Tıbbi Onkoloji Kliniği'ne gelen ,hem metastaz gelişmesi, hem de radyoaktif iyot tedavisine refrakter olması nedeniyle sistemik tedavi amacıyla yönlendirilmiş olan 57 metastatik tiroid kanseri olan hastaların dosyaları retrospektif olarak analiz edildi.

Bulgular : Hastaların, %52'si (30 hasta) kadın, %48'i (27 hasta) erkek, yaş ortalaması 57,11, ve tamamı 18 yaşın üzerinde idi. Hastaların, %59,8'i (35 hasta) iyi diferansiye tiroid kanseri, %29,8'i (17 hasta) medüller tiroid kanseri, %5,3'ü (3 hasta) anaplastik tiroid kanseri, % 3,5'i (2 hasta) az diferansiye kanseri ve %1,8'i (1 hasta) medüller-papiller senkron kanseri idi. Birinci seri sistemik tedavi yanıtı tüm 57 olguda değerlendirildiğinde progresyonsuz sağ kalım (PFS) süresi kemoterapi ve sorafenib için sırasıyla 4,25 ve 6,33 ay olarak tespit edildi (P:0.035). İkinci seride toplam 57 hasta dosyası retrospektif olarak değerlendirildi ve PFS, kemoterapi alan ve sorafenib kullanılan hastalarda 4,1 ve 7,77 ay olarak bulundu (P<0.001).

Sonuç: Radyoaktif iyot tedavisine refrakter olarak diferansiye tiroid kanserleri ve medüller tiroid kanserleri tedavisinde tirozin kinaz inhibitörü kullanılmaktadır. Tiroid kanserlerinde lenvatinib, sorafenib ve vandetanibin progresyonsuz sağkalım üzerine olan etkisi sistemik kemoterapiden üstün bulunmuştur. Özellikle, ülkemizde olmak üzere radyoaktif iyot tedavisine refrakter iyi diferansiye tiroid kanserlerinde sorafenib, etkinlik ve toksisite profili açısından tercih edilebilecek bir sistemik tedavi seçeneğidir.

Anahtar kelimeler: Tiroid, Kanser, Sorafenib

Introduction

Thyroid cancer is the most common type of endocrine cell-derived cancer. Incidence of thyroid cancer increases with

increasing age due to the various environmental and genetic factors. When considering all age groups, average incidence of thyroid cancer is 6.5% in women and 5.4% in men [1]. In general, about 75% of malignant thyroid tumours are presented as papillary thyroid cancer and 13% as follicular thyroid cancer, while the other types of thyroid cancer occur as medullary thyroid cancer by 7-8%, and to a lesser extent, as anaplastic (undifferentiated) thyroid cancer [2]. Recently, there have been some changes in the incidence order of thyroid cancer and particularly, micropapillary variant has been started to be observed more commonly [3]. In papillary thyroid cancers, the presence of RAS oncogene and p21 are associated with higher frequency of lymph node involvement, whereas the presence of GS-alpha mutation is more commonly associated with distant metastasis [4]. Administration of radioactive iodine is important in the treatment of differentiated thyroid cancers [3]. External radiotherapy is also recommended in patients aged over 45 years who have papillary thyroid cancer with a large mass and a large volume load after surgery [5]. Most recent approaches in medication therapy of thyroid cancers are chemotherapy, which is under phase II investigations, and tyrosine kinase inhibitors, for which are under phase III investigations [6]. A mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-AKT the mammalian target of rapamycin (mTOR) are active growth pathways in thyroid cancer; in addition, BRAF V 600 E mutation is tested positive in 40% of primary cancers and 60-70% of metastatic cancers. RAS family is more active in follicular thyroid cancer, while rearranged during transfection (RET) pathway is more active in medullary thyroid cancer [7]. Most commonly used tyrosine kinase inhibitors are lenvatinib, sorafenib, vandetanib and cabozantinib [8]. In our current study, we evaluated the patients who received sorafenib, doxorubicin, cisplatin, cisplatin-doxorubicin, cisplatin-etoposide, carboplatin-paclitaxel and other therapies. We aimed to determine the efficacy of systemic treatment especially in patients with metastatic thyroid cancer.

Materials and Methods

A retrospective evaluation was conducted for a total of 57 patients with complete thyroid cancer. Data were collected and evaluated regarding patient age, gender, age at cancer diagnosis, time from first diagnosis to systemic treatment. Fluro-D-glucose-positron emission tomography/computed tomography (FDG-PET/CT), magnetic resonance

(MR), computed tomography (CT), ultrasonography (USG) imagings, serum calcitonin, serum thyroglobulin, carcinoembryonic antigen (CEA) markers and histological cancer types were evaluated for progression free survival (PFS) time analysis. Patients were grouped according to the treatments they received. Also patients who received chemotherapy were grouped separately according to chemotherapy regimen. The study was approved by the Institutional Ethics Committee (No: 2018-17/17) and performed in accordance with the Declaration of Helsinki.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). IBM SPSS version 20 (SPSS, Inc., Chicago, IL,USA) was used for statistical analyses. Descriptive statistical analyses (mean, median, minimum, maximum, standard deviation) were performed. Demographic data were presented as minimum, maximum,± standard variation. Survival data were analysed using Kaplan–Meier curves. Statistical analysis was performed using log-rank test. A P value of < 0.05 was considered to be statistically significant.

Results

Medical records of 57 patients with metastatic thyroid cancer, referred for systemic therapy were retrospectively analysed. 52% (n:30) of the cases were women and 48% (n:27) were men, and the mean age was 57.11 years. 59.8% (n:35) of the cases were diagnosed with well differentiated thyroid cancer, 29.8% (n:17) with medullary thyroid cancer, 5.3% (n:3) with anaplastic thyroid cancer, 3.5% (n:2) with poorly differentiated thyroid cancer and 1.8% (n:1) with medullary-papillary cancer (Table 1). Response to first line systemic therapy was evaluated in 57 patients. 35 of these patients were diagnosed with differentiated thyroid cancer. 28.1% of the patients with thyroid cancer received treatment with doxorubicin, 28.1% with cisplatin-doxorubicin, 10.5% with cisplatin-etoposide, 14% with carboplatin-paclitaxel and 10.5% with sorafenib (Table II). Mean progression-free survival (PFS 1) was 4.25 months in patients treated with first line chemotherapy and 6.33 months in patients treated with sorafenib (P:0.035) (Table III). For the second line systemic therapy, a total of 57 patient records were retrospectively evaluated. 1.8% of the cases received treatment with doxorubicin, 8.8% with cisplatin, 54.4% with sorafenib, 7% with cisplatin–doxorubicin, 12.3% with

carboplatin-paclitaxel, 5.3% and 10.5% with other therapy (Table IV). PFS among patients with thyroid cancer who received chemotherapy was 4.1 months, while treatment with sorafenib resulted in a PFS of 7.77 months ($P<0.001$) (Table V). Initial thyroid surgery was total thyroidectomy in 94% of the patients (Table VI).

Table I. Histopathological subtypes of thyroid cancer

Subtypes of thyroid cancer	Frequency (n=57)	Percent (%)
Classical papillary	20	35.1%
Tall cell	1	1.8%
Follicular	5	8.8%
Hurthle	3	5.3%
Medullary	17	29.8%
Anaplastic	3	5.3%
Follicular variant of papillary	2	3.5%
Insular follicular variant of papillary carcinoma	3	5.3%
Poorly differentiated	2	3.5%
Medullary and Papillary	1	1.8%
Total	57	100.0%

Table II. Surgery types

Surgery types	Patients (n=57)	Percent (%)
Total	54	94.7%
Subtotal Neck Dissection	2	3.5%
No surgery	1	1.8%
Total	57	100.0%

Table III. First-line agents and administration frequencies

First-line agents	Frequency	Percent (%)
Doxorubicin	16	28.1
Cisplatin	5	8.8
Sorafenib	6	10.5
Cisplatin Doxorubicin	16	28.1
Carboplatin Paclitaxel	8	14.0
Cisplatin Etoposide	6	10.5
Total	57	100.0

Table IV. Second-line agents and administration frequencies

Second-line agents	Patients	Percent (%)
Doxorubicin	1	1.8
Cisplatin	5	8.8
Sorafenib	31	54.4
Cisplatin Doxorubicin	4	7.0
Carboplatin Paclitaxel	7	12.3
Vandetanib	3	5.3
OTHER CT	6	10.5
Total	57	100.0

Table V. Progression-free survival (PFS) in first-line therapy

First-line therapy	Patients	Mean (months)
Doxorubicin	16	3.88
Cisplatin	5	3.00
Sorafenib	6	6.33
Cisplatin Doxorubicin	16	4.06
Carboplatin Paclitaxel	8	4.75
Cisplatin Etoposide	6	4.00
Total	57	4.25

Table VI. Progression-free survival (PFS) in second-line therapy

Second-line therapy	Patients	Mean (months)
Doxorubicin	1	5.00
Cisplatin	5	4.20
Sorafenib	31	7.77
Cisplatin Doxorubicin	4	3.00
Carboplatin Paclitaxel	7	4.14
Vandetanib	3	3.33
OTHER CT	6	3.50
Total	57	5.95

Discussion

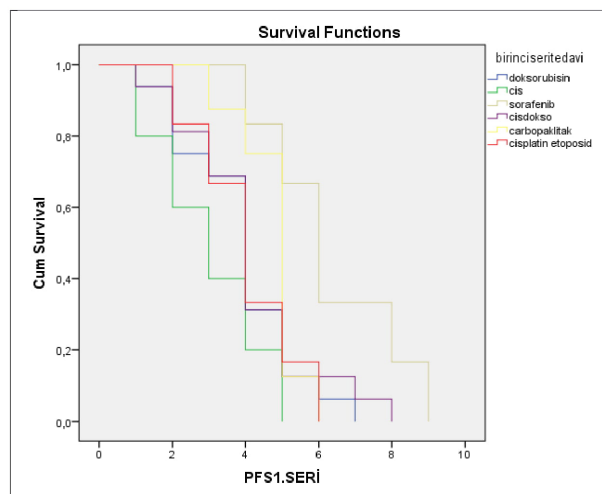
The efficacy of tyrosine kinase inhibitor in patients with thyroid cancer is the mainstay of our treatment algorithm. A study conducted with doxorubicin for the treatment of metastatic thyroid cancer in 30 patients showed partial and stable responses in three-fifths of patients who histologically diagnosed with medullary carcinoma, one-third of patients with papillary carcinoma and two-fifths of patients with Hurthle cell carcinoma [9].

A randomised evaluation was conducted for efficacy and toxicity of doxorubicin alone and combination of cisplatin and doxorubicin in patients with advanced thyroid carcinoma. 92 patients were included, of which 84 were evaluated. Histological classification was made according to Eastern Cooperative Oncology Group (ECOG) performance status and metastatic sites. Forty one patients received doxorubicin as a single agent and seven patients (17%) showed partial response. Forty-three patients received combination therapy resulting in five complete and six partial responses (combined response rate, 26%). This difference was not significant for overall response rate ($P>0.1$). Four of five patients who showed complete response survived for more than 2 years. None of the patients with partial responses survived for more than 2 years. Life-threatening toxicities caused by chemotherapy occurred in five patients treated with drug combination and two patients treated with doxorubicin alone.

Moreover, side effects were higher with the combination therapy [10]. In a phase II study which included all thyroid cancers treated with epirubicin and carboplatin, 1 patient achieved complete response and 5 patients achieved partial response [11]. In the present study, mean PFS 1 was 4.25 months in subjects receiving chemotherapy and 6.33 months in subjects receiving tyrosine kinase inhibitor during first-line therapy of thyroid cancer, while PFS 2 was 4.1 months in subjects receiving chemotherapy and 7.77 months in subjects receiving tyrosine kinase inhibitor during second-line therapy. Abramson et al. conducted a study on sorafenib therapy in 30 patients with thyroid cancer, of which 27 were diagnosed with differentiated thyroid cancer, and found a clinical benefit rate of 77%, partial response rate of 23% and stable disease rate of 53%, while the mean time to relapse of metastatic disease was 18 months [12]. In another phase II study of sorafenib in 41 patients with papillary thyroid cancer, Kloos et al., found a clinical benefit rate of 56%, partial response rate of 15% and stable disease rate of 41%, and reported the mean time to relapse of metastatic disease as 15 months [13]. In efficacy assessment of sorafenib in our study, time to relapse of metastatic disease was found to show a statistical benefit ($P < 0.035$) (Figure 1) at 6.33 months during first-line therapy and also found to be statistically significant ($P < 0.001$) (Figure 2) at 7.77 months during second-line therapy with sorafenib. A phase III study conducted for radioactive iodine refractory metastatic thyroid cancers, for which sorafenib was approved and 92% of which consisted of differentiated thyroid cancer showed a median disease-free survival of 10.8 months and 5.8 months in sorafenib and placebo groups, respectively ([HR] 0.59% 95% CI 0.45-0.76; $P < 0.0001$) [14]. The most important reason for difference in treatment response in the present study compared to other studies is the delayed treatment approval of tyrosine kinase inhibitors and the requirement of previous use of chemotherapy by Social Security Institution. In SELECT study, a phase III trial of lenvatinib, median PFS in radioactive iodine-refractory metastatic thyroid cancer was 18.3 months, which is about two times longer than PFS achieved with sorafenib [15]. This study has some limitations. This study represents a trial which evaluated the effects of tyrosine kinase inhibitors and conventional chemotherapy on thyroid cancer in a population cohort of 57 patients without a control group. It is a two centre study conducted with a small sample size. There is a possibility for bias in results obtained; this was a retrospective study and there is a potential for selection bias during retrospective data collection process. Although, we recorded the detailed data, a prospective study would provide a better evaluation for tyrosine kinase

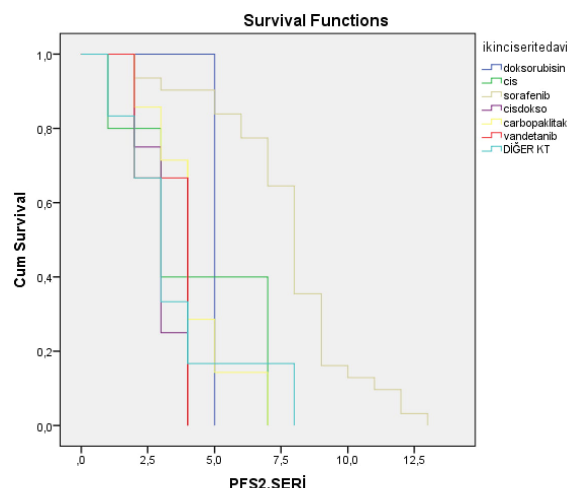
inhibitors and conventional chemotherapy in patients with thyroid cancer. Therefore, performed evaluations must be conducted in a larger patient group.

In conclusion, standard treatment for thyroid cancer is multikinase inhibitors. While, a tyrosine kinase inhibitor called vandetanib and a multikinase inhibitor called lenvatinib are among the standard treatments in medullary thyroid cancer, these are not authorised and used in our country because of the challenges in reimbursement in Turkey. Therefore, the best treatment in radioactive iodine-refractory metastatic differentiated thyroid cancer is a tyrosine kinase inhibitor called sorafenib.



Cis: Cisplatin, cisdokso: Cisplatin -Doxorubicin, Carbopaklitak: Carboplatine- Paclitaxel

Figure 1. 1st progression free survival (PFS) time



Cis: Cisplatin, cisdokso: Cisplatin -Doxorubicin, Carbopaklitak: Carboplatine- Paclitaxel

Figure 2. 2nd progression free survival (PFS) time

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Effects of carbachol on apoptosis in human chronic myelogenous leukemic K562 cell line

İnsan kronik miyeloid lösemi K562 hücrelerinde karbakolün apoptoza etkisi

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ABSTRACT

Objectives: Muscarinic receptors mediate diverse actions of acetylcholine in the central nervous system and in non-nervous tissues innervated by the parasympathetic nervous system. Our study aims to evaluate the potential association of the M₃ muscarinic receptor with K562 cell proliferation and death.

Materials and Methods: Cell proliferation was evaluated by bromodeoxyuridine (BrDU) incorporation. To show early, late apoptosis and cell death, cells were labelled with Annexin V, propidium iodide (PI) and analyzed by flow cytometry. Nuclear extracellular signal-regulated kinase (ERK/pERK) expression was measured by western blot analysis.

Results: Treatment with carbachol (CCh) for 48h decreased cell number. Exposing K562 cells to CCh for 24h decreased the number of early apoptotic cells but did not change the number of late apoptotic and necrotic cells. CCh treatment for 48h increased the number of necrotic cells, but decreased the number of early and late apoptotic cells. In response to CCh, nuclear ERK expression was increased and this effect was reversed by 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4DAMP). Nuclear pERK expression was decreased in CCh treated cells, 4DAMP did not reverse the effect.

Conclusion: Our data suggest that cholinergic agonist CCh affects cell proliferation in K562 cells not only through muscarinic receptors but also through other cholinergic receptors.

Keywords: Muscarinic receptors, K562 cells, Carbachol, Cholinergic system

ÖZ

Amaç: Muskarinik reseptörler merkezi sinir sisteminde asetilkolinin çeşitli etkilerine aracılık ettiği gibi, parasempatik sinir sistemi ile etkileşen sinirsel olmayan dokulara da aracılık ederler. Çalışmamızda, M₃ muskarinik reseptör alttipinin K562 kanser hücre çoğalması ve ölümündeki rolünü belirlemeye çalıştık.

Gereçler ve Yöntemler: Hücre çoğalması bromodeoksüridin (BrDU) yöntemi ile belirlenmiştir. Hücre ölümü, erken ve geç apoptoz Annexin V ve propidyum iodid (PI) varlığında akış sitometrisi yöntemi ile gösterilmiştir. Nükleer dış sinyal düzenleyici kinaz (ERK/fosfo-ERK) ekspresyonu western emdirimi yöntemi ile belirlenmiştir.

Bulgular: Çalışmamızda 48 saat karbakol(CCh) ile muamele edilen K562 hücre sayısında azalma belirlenmiştir. Hücreler 24 saat CCh ile muamele edildiklerinde erken apoptotik hücre sayısında azalma gözlemlenirken geç apoptoz ve nekrotik hücre sayısında değişim olmamıştır. Bununla birlikte, 48 saat boyunca CCh ile muamele olan hücrelerde, erken ve geç apoptotik hücre sayısı azalırken, nekrotik hücrelerin sayısı artmıştır. CCh ile muamele edilen hücrelerde nükleer ERK ekspresyonu artarken bu etki 1,1-dimetil-4-difenilasetoksipiperidinium iodid (4DAMP) ile geri çevrilmiştir. Aynı koşullarda CCh ile muamele edilen hücrelerde nükleer pERK ekspresyonu azalmış, bu etki 4DAMP ile geri çevrilmemiştir.

Sonuç: Bulgularımız, K562 hücre proliferasyonundaki kolinerjik etkinin yalnızca muskarinik mekanizma ile değil diğer kolinerjik reseptörlerin de katkısıyla gerçekleştiğini düşündürmektedir.

Anahtar kelimeler: Muskarinik reseptör, K562 hücreleri, Karbakol, Kolinerjik sistem

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Introduction

G-protein-coupled receptors (GPCRs), the largest family of cell-surface molecules involved in signal transmission, have recently emerged as crucial players in tumour growth and metastasis. These receptors control key physiological functions, including neurotransmission, hormone and enzyme release from endocrine and exocrine

glands, immune responses, cardiac – and smooth-muscle contraction and blood pressure regulation, just to name a few. Their dysfunction contributes to some human diseases; therefore, GPCRs represent the target of 50–60% of all current therapeutic agents, either directly or indirectly [1].

Five subtypes (M_1 - M_5) of receptors with seven transmembrane segments are integral membrane proteins, bind with acetylcholine (ACh) in the extracellular segment, and thereafter interact with and activate GTP-binding regulatory proteins (G proteins) in the intracellular segment [2]. Three muscarinic receptor subtypes (M_1 R, M_3 R, and M_5 R) stimulating cellular signaling are conditional oncogenes when expressed in cells capable of proliferation [3]. Many studies have shown that cholinergic agonist carbachol (CCh) induced cancer cell proliferation [4,5].

Leukemia is a clonal disorder characterized by blocked normal differentiation and cell death of hematopoietic progenitor cells. Chronic myelogenous leukemia (CML), is a hematopoietic stem cell disorder with increased production of granulocytes at all stages of differentiation, leading to a myeloproliferative syndrome [6,7]. The K562 cell line derived from a CML patient during blast crisis was examined for properties of B and T lymphocytes and cell lines. Although K562 cells have some T cell properties, these are not exclusive [8]. K562 cell lines are good models for studying cell proliferation and apoptosis in CML. We have previously demonstrated that CCh decreased proliferation of K562 cells supplemented after starvation with 1% serum in 24h, an effect prevented by atropine [9].

Apoptosis is one of the ways for cell death that is referred to as programmed cell death (PCD). It plays important roles in embryonic development, immune system maturation and cytotoxic effector function, and carcinogenesis [10]. Apoptosis is executed by two pathways. Death receptors, such as Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) DR4 and DR5 trigger the extrinsic/death receptor pathway [11]. The response to radiation or cytotoxic drug-induced cellular stress activates the intrinsic/mitochondria-dependent pathway [12]. The development of new therapies for the treatment of cancer has been possible by studying apoptotic processes [13].

Programmed cell death uses adenosine triphosphate (ATP), synthesizes new RNA and protein and thus forms active cellular suicide. PCD activates endogenous endonucleases that degrade the cell's DNA. Thereafter, the genetic template required for cellular homeostasis is destroyed [14]. However, there are various ways for cell death, for example hypoxia and exposure to certain toxins cause the form of cell death termed “necrosis”.

Increased membrane permeability, cell swelling, and rupture are the early events; whereas, loss of plasma membrane integrity is a relatively late event in PCD. PCD seems to play an important role in several physiological situations [15].

In neurons, it has been shown that muscarinic receptors act via activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) which are referred to as mitogen-activated protein (MAP) kinase 1 [16]. M_3 R has an important role in the endogenous and exogenous ACh-induced cell proliferation and phosphorylation of ERK and AKT in gastric cancer cells [17].

Materials and Methods

Carbachol, 4DAMP, atropine, Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Sigma Chemical Co, St. Louis, MO, USA. Fetal bovine serum (FBS) was obtained from Biol. Ind. (Beit Haemek, Israel). Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-indolyl – phosphate (NBT/BCIP) were purchased from Promega (Madison, WI, USA). phosphorylated ERK (pERK), β -actin antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies were purchased from Sigma (St Louis, MO, USA).

Cell culture

K562 cells were grown in suspension using RPMI medium supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ humidified atmosphere. Cells were usually seeded at a density of 10⁵ cells/ml and one half of the medium was replaced every 3-4 days. These cells were then starved by seeding into flasks containing RPMI-1640 medium with 0% FBS. Afterwards, these “starved cells” were placed into a medium containing 1% serum, with and without 100 μ M CCh, 10 μ M 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4DAMP), 10 μ M atropine for different times.

Cell counting

The change in K562 cell count in response to CCh stimulation was evaluated by assessing proliferation and cell viability by the trypan blue exclusion test and bromodeoxyuridine (BrDU) labeling, respectively. The contribution of muscarinic receptors was investigated by using the non-specific muscarinic antagonist, atropine. Cell counting was carried out using an ELISA reader (Multiskan Microplate Reader, Thermo Scientific, USA). K562 cells were treated with 0-100 μ M CCh and/or antagonist atropine (10 μ M) for 48h and aliquots were

removed at indicated times. Antagonist was added 30 min prior to CCh. The average of 3 experiments performed in duplicate was used for data analysis.

Flow cytometric analysis

In order to detect early and late apoptosis and cell death, cells were stained with FITC conjugated Annexin V and propidium iodide (PI) according to the manufacturer's instructions. Samples were then analyzed by flow cytometry (BD FACS Canto, Becton, Dickinson and Company, USA).

Western blot analysis

K562 cells were treated with 100 μ M CCh and/or M₃R-selective antagonist 4DAMP (10 μ M) for 5 minutes. The duration of CCh treatment was determined by our previous studies (unpublished data). Antagonist, 4DAMP (10 μ M) was added 30 min prior to CCh. After addition of CCh and 4DAMP, cells were washed with phosphate-buffered saline (PBS) and were later frozen at -80°C until further treatment. The frozen cells were homogenized in ice-cold 10 mM Tris-HCl (pH 7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1 g/ml leupeptin, 1 M pepstatin, 10 g/ml soybean trypsin inhibitors) with a 9-gauge needle. The samples were centrifuged at $300\times g$ for 5 min at 4°C . The resulting supernatant was centrifuged at $13000\times g$ for 20 min at 4°C . The pellets were resuspended and washed twice in the same buffer and stored at -80°C . The protein content of pellets was determined by the Lowry method [18]. 50 μ g of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 m, Germany). The membranes were blocked at room temperature for 60 min. Later the membranes were incubated overnight at 4°C with antibodies against ERK and pERK (1/500). The blots were washed with TBS containing 0.05% Tween-20 (TBS-T) and were later incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20°C). The antibody-antigen complex was detected with nitra blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). The densitometric analyses were carried out with Bio-Rad Molecular Analyst software (free edition, www.totallab.com).

Results

Effect of CCh on K562 cells proliferation

In this study, we showed that there was a decrease in cell number in the group treated with 100 μ M CCh for 48h when

compared to the control group. However, this decrease was not reversed by atropine treatment (Figure 1).

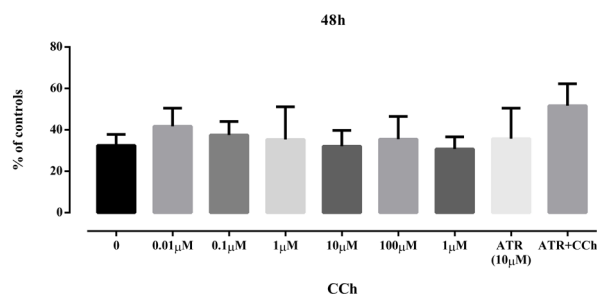


Figure 1. Effect of CCh on K562 cells proliferation. K562 cells were treated with 0-100 μ M CCh, 10 μ M atropine, 100 μ M CCh and 10 μ M atropine for 48 hours. Control cells were not treated with CCh. The results were shown as \pm SEM by taking the average of 3 experiments. ATR: Atropine.

Effect of CCh on K562 cells apoptosis

We demonstrated that after exposing K562 cells to 100 μ M CCh for 24 h the number of early apoptotic cells was decreased by $\sim 10\%$ but no change was observed in the number of late apoptotic and necrotic cells. After treatment with CCh for 48h, the number of necrotic cells was increased by 1.5-fold whereas apoptotic cells were decreased by ~ 1.55 -fold compared to control cells (Figure 2).

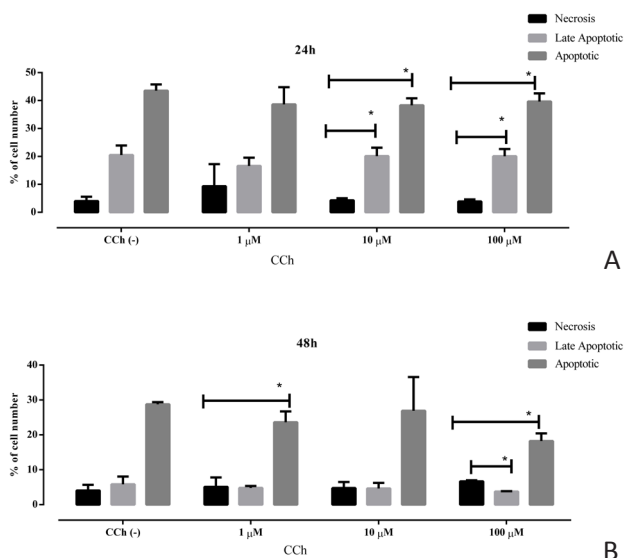


Figure 2. Effect of CCh on K562 cells apoptosis. K562 cells were treated with 0-100 μ M CCh for (A) 24h and (B) 48h. Control cells were not treated with CCh. The results were shown as \pm SEM by taking the average of 3 experiments. *P < 0.05 for significant change compared with the CCh (-) group.

Effect of CCh on nuclear ERK expression

In cells treated with CCh for 5 min, nuclear ERK expression was increased by 14% compared to the control, but this increase was found to be 3% in cells treated with 4DAMP prior to CCh (Figure 3). On the other hand, in cells treated with CCh for 5 min, expression of nuclear pERK was reduced by 22% compared to the control, however this effect was not antagonized by 4DAMP (Figure 4).

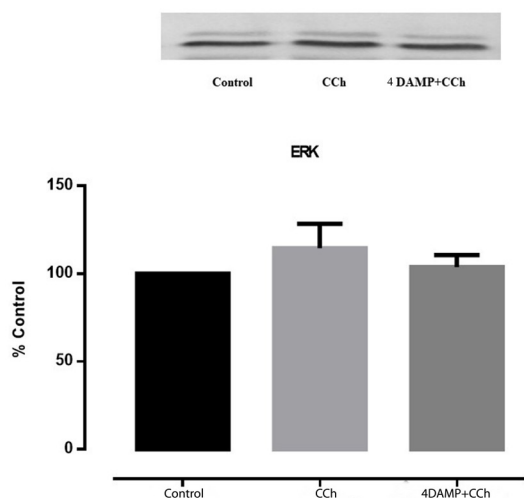


Figure 3. Effect of CCh on nuclear ERK expression. K562 cells were treated with 100 μ M CCh for 5 min. Antagonist, 4DAMP (10 μ M) was added 30 min prior to CCh. Control cells were not treated with CCh. Percent increase in ERK expression relative to control in cells. The results were shown as \pm standard error (SD) by taking the average of 3 experiments.

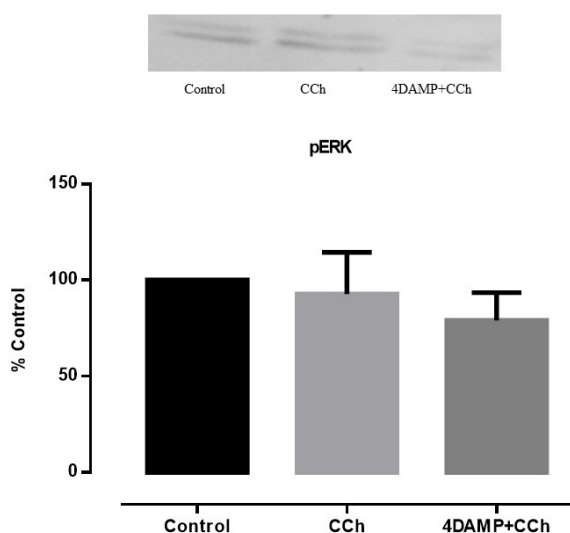


Figure 4. Effect of CCh on nuclear pERK expression. K562 cells were treated with 100 μ M CCh for 5 min. Antagonist, 4DAMP (10 μ M) was added 30 min prior to CCh. Control cells were not treated with CCh. Percent increase in pERK expression relative to control in cells. The results were shown as \pm standard error (SD) by taking the average of 3 experiments.

Statistical analysis

All data were expressed as mean \pm SEM. Multiple t tests were used for the analysis of data. For all statistical calculations, significance was considered to be a value of $P < 0.05$.

Discussion

Acetylcholine serves as a neurotransmitter both in the central and the peripheral nervous systems, where it controls functions including muscle contraction, neurotransmission among others. Recent studies demonstrating that ACh also regulates cell proliferation [19] and apoptosis [20] initiated research on the role of nAChRs and mAChRs in the development and progression of cancer and in stem cell physiology. Based on these studies, we asked whether the M_3 R subtype may affect the proliferation of the K562 cell line.

Muscarinic receptor agonists stimulate cell proliferation, survival, migration, and invasion, as shown by *in vitro* studies using human colon cancer cells. These effects are regulated by complex mechanisms involving interacting post- M_3 R signaling pathways which activate post-receptor signaling cascades [21]. Colon cancer cell proliferation is regulated by rapid, reversible activation of ERK1/2; whereas, cell survival and resistance to radiation is regulated by PI3K/AKT activation [21,22]. M_3 R activation stimulates colon cancer growth in animal models relevant to human colon cancer [23]. Similarly, M_3 R deficiency attenuates tumor formation [24,25]. Thus, M_3 R expression and activation seem to play important roles in the progression of colon neoplasia. [26].

Expression of mRNAs of muscarinic receptors (M_2 , M_3 and M_4) has been shown in K562 cells by using reverse transcription polymerase chain reaction and immunoblotting, but the roles of muscarinic receptors have not been clarified yet [27,28]. There is previous evidence suggesting that muscarinic receptors can regulate cell proliferation depending on the growth context of the cell. Whether cells are in a quiescent state or growing appear to determine the type of effect. Our previous data showed that *in vitro* proliferation of K562 cells was thoroughly dependent on the presence of fetal bovine serum that contained various growth factors. Treating serum-deprived K562 cells with a cholinergic agonist, CCh, a choline ester, led to a significant increase in DNA synthesis, implying the roles of cholinergic receptors in cell growth [9]. On the other hand, CCh produced a decrease in DNA synthesis in

K562 cells supplemented with 1% fetal bovine serum after starvation. We also demonstrated that phospholipase C and intracellular calcium were involved in CCh-mediated inhibition of proliferation in K562 cells [29]. These findings lend support to the hypothesis that ACh or CCh can generate intracellular effects through their action on cholinergic receptors. Our previous study demonstrated that CCh also enhanced NO production in K562 cells [9, 28]. The current study showed that treatment with CCh for 48h decreased the cell number, indicating that CCh had a very fast and irreversible effect to promote cells to necrotic cell death. Supporting this hypothesis, decrease of cell number was not reversed by atropine treatment. Exposing K562 cells to 100 μ M CCh for 24 hours decreased the number of apoptotic cells, possibly because of its promoting effect on necrosis. Treatment with 100 μ M CCh for 48 hours increased the number of necrotic cells whereas apoptotic cells were decreased compared to control cells (Figure 2). The increase in the necrotic cell number in 48 hours appeared to be compatible with decrease in cell proliferation.

Kodaira and his colleagues show that in gastric cancer, ERK signaling is observed following muscarinic receptor activation. However, the failure of ERK signaling to stimulate gastric cancer cell proliferation raises questions regarding the importance of this observation [30]. Expression of muscarinic receptors, ChAT (choline acetyltransferase), and CrAT (carnitine acetyltransferase), and ACh production, have been reported in human leukemia cell lines [31,32]. Shah and his colleagues show that muscarinic receptors and ligands play a major role in cancer, and then suggest that these possible carcinogens are an additional matter to investigate and environmental effects need to be addressed [33]. CCh affects cell proliferation via cholinergic receptors through ERK signaling [34]. In our experiments we demonstrated that ERK expression increased overall in response to CCh treatment, however, phospho-ERK expression was reduced when compared to control (Figure 4). Therefore, we suggest that increase in ERK indicates a cellular survival reflex of K562 cells, whereas CCh reduces the phosphorylation of ERK, which would otherwise promote proliferation.

In conclusion, our results support the notion that the cholinergic agonist CCh may have roles in cell death and affect cell proliferation by activating not only muscarinic receptors but also other signaling pathways. Future studies addressing a possible role for nicotinic acetylcholine receptors merit investigation.

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The evaluation of complications and mortality in non-alcoholic steatohepatitis-related cirrhosis

Non-alkolik steatohepatite bağlı sirozda komplikasyonların ve mortalitenin değerlendirilmesi

Sezgin VATANSEVER, Zehra Betül PAKÖZ

ABSTRACT

Objectives: Cirrhosis is seen in 4-8% of patients with non-alcoholic fatty liver disease (NAFLD), and death occurs in 1-5% of them due to hepatocellular carcinoma (HCC). The aim of this study was to determine the factors associated with complications and mortality in patients with cirrhosis secondary to non-alcoholic steatohepatitis (NASH).

Materials and Methods: The patients with cirrhosis due to NASH diagnosed between 2008 and 2018 in our clinic formed the study population. Patients with diabetes, obesity, or insulin resistance and those with cirrhosis due to other causes were excluded. The patients were enrolled and followed up prospectively.

Results: A total of 185 patients were included in the study. The survival was 94.6% at the 1st year and 57.0% at the 5th year. Median survival duration was 5.83 years. The rate of HCC development was 0.7% at the 1st year and 9.7% at the 5th year. In the multivariate Cox analysis, age (OR: 1.12, 95% CI: 1.04-1.21; P = 0.003), creatinine (OR: 24.4, 95% CI: 2.32-257.8; P= 0.008) and encephalopathy (OR: 24.49, 95% CI: 1.06-19.6; p = 0.042) were found as independent predictors of mortality. Development of ascites occurred in 46.9%, variceal bleeding in 21.9% and encephalopathy in 18% of patients at the 5th year.

Conclusion: Patients with NASH-related cirrhosis should be carefully monitored for HCC development, variceal bleeding, ascites, and encephalopathy.

Keywords: Non-alcoholic Steatohepatitis, Cirrhosis, Mortality

ÖZ

Amaç: Non-alkolik yağlı karaciğer hastalığı (NAYKH) saptanan hastaların %4-8'inde siroz ve %1-5'inde hepatoselüler karsinom (HCC) nedeni ile ölüm görülmektedir. Bu çalışmada amaç, non-alkolik steatohepatit (NASH)'e sekonder siroz gelişen hastalarda, siroza ait komplikasyonlar ve mortalite ile ilişkili faktörlerin belirlenmesidir.

Gereçler ve Yöntemler: Çalışmaya, 2008-2018 yılları arasında kliniğimizde NASH'e bağlı siroz tanısı alan hastalar alındı. Diyabeti, obezitesi ya da insülin direnci olan ve diğer siroz nedenleri dışlanan hastalar NASH'e bağlı siroz olarak kabul edildi. Hastalar prospektif olarak kayıt altına alınıp takip edildi, analizler retrospektif olarak yapıldı.

Bulgular: Çalışmaya toplam 185 hasta dahil edildi. Sağkalım 1. yılda %94,6 ve 5. yılda %57,0 bulundu. Median sağkalım 5,83 yıl saptandı. HCC'un ortaya çıkması 1. yılda %0,7 ve 5. yılda %9,7 bulundu. Çok değişkenli Cox analizinde yaş (OR 1,12, 95% CI 1,04-1,21; P=0.003), kreatinin (OR 24,4, %95 CI 2,32-257,8; P=0.008) ve ensefalopati (OR 24,49, %95 CI 1.06-19.6; p=0.042) bağımsız prediktörler olarak saptandı. Hastalarda 5. yılda asit gelişimi %46,9, varis kanaması gelişimi %21,9 ve ensefalopati gelişimi %18 saptandı.

Sonuç: Non-alkolik steatohepatite sekonder siroz gelişen hastalar HCC, varis kanaması, asit ve ensefalopati gelişimi açısından dikkatli takip edilmelidir.

Anahtar kelimeler: Non-alkolik steatohepatit, Siroz, Mortalite

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common disease of the liver in recent years and affected about 25% of adults globally [1]. Cirrhosis develops in 4-8% of patients with NAFLD and the mortality rate due to NAFLD-induced HCC is 1-5%. NAFLD is one of the most common causes of liver transplantation and HCC in the United States (US) [2,3]. Among all causes of transplantation in the US, non-alcoholic steatohepatitis (NASH) takes place in 1.2% of all causes in 2001, which

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has risen to 9.7% in 2009 [4]. In the US, it is estimated that there are 64 million cases in 2017 that yielded an economic cost of 103 billion \$ [5].

There is a close association between NASH and type 2 diabetes mellitus (DM), central obesity, dyslipidemia, the metabolic syndrome. The prevalence of NAFLD has been reported to be 40-70% in type 2 DM patients and up to 90% in obese individuals [6,7]. In parallel with the increased prevalence of obesity, the prevalence of NASH increased from 15% in 2005 to 25% in 2010 [4]. As the prevalence of obesity and type 2 DM has increased in society, NASH-related liver disease is estimated to increase by 178% in the year 2030 [8]. Although, cardiovascular diseases are the most common cause of mortality in NASH patients, the presence of hepatic fibrosis is a significant prognostic predictor [9]. The hazard ratio was found 1.9 in F0 fibrosis stage and 104.9 in F4 for the development of the severe liver disease. The advanced fibrosis stage is the most critical parameter in predicting overall mortality [10].

Risk factors for advanced liver disease include age, increased body mass index and the presence of DM [11]. The progression of fibrosis in NASH is slower than other chronic liver disease causes. The mean duration to develop a severe hepatic disease is 22-26 years in patients with stage F0, 9.3 years in stage F2 and 0.9 years in stage F4 [9]. In the presence of NASH in NAFLD, 7 years are required for each fibrosis stage, whereas the period is 14 years unless NASH exists.

In NAFLD, fibrosis begins in the pericellular space around the central vein and the peri-sinusoidal region. Therefore, portal hypertension in patients with NAFLD begins before cirrhosis. Portal hypertension complications such as esophageal variceal bleeding are the most common cause of first referral to hospital in these patients [13].

NASH-related HCC usually develops in elderly patients with advanced fibrosis stage which is less aggressive than HCC due to viral hepatitis. Therefore, it can mistakenly be overlooked during routine imaging studies [14].

The aim of this study was to determine cirrhosis-induced complications and independent factors associated with mortality in patients with NAFLD-induced cirrhosis.

Materials and Methods

The patients who were diagnosed as NAFLD-induced cirrhosis between 2008 and 2018 in our clinic were

enrolled to the study. The patients with DM, or insulin resistance, obese patients and those who had cirrhosis due to other causes were excluded. Viral hepatitis, autoimmune hepatitis and Wilson's disease had been excluded during the diagnostic process in every patient. The patients were enrolled and followed up prospectively. Statistical analysis was performed at the end of enrollment retrospectively.

The patients were diagnosed as cirrhosis by clinical and biochemical examinations together with imaging modalities. Cirrhosis, jaundice, ascites, hepatic encephalopathy, prothrombin time (PT) prolongation, the presence of low serum albumin and presence of nodular liver and splenomegaly on radiological examinations were considered as signs of chronic liver disease.

In addition, patients who were diagnosed two years before or longer, and presented with a mass in the liver were not included in the study.

The patients who had ascites were offered a salt-free diet, and spironolactone or spironolactone plus furosemide were also given orally. Propranolol 40-80 mg/day was started in patients with varices on endoscopy. Band ligation was performed to patients who had variceal bleeding or patients with grade 2-3 varices on endoscopy. Patients who had encephalopathy were given low protein diet and lactulose and rifaximine 600 mg/day was added to treatment when encephalopathy recurred. Obese patients were advised to lose weight and diabetic patients were advised for blood glucose regulation. Patients with cirrhosis-related complications were referred to liver transplantation. The patients were followed-up at 6-12 months intervals. The study was approved by Izmir Katip Celebi University Ethics Committee (No:2018-394).

Statistical Analysis

Statistical analysis of the data was done with the help of SPSS version 22.0 package program. Normally distributed numerical data were expressed as mean and standard deviation. The chi-square test was used for categorical variables. The normality and homogeneity of the groups were evaluated. Mann Whitney U test was used for data not consistent with normal distribution, while Student T was used for data fitting normal distribution. The emergence of complications of cirrhosis was demonstrated by Kaplan-Meier graph. Cox regression univariate analysis was performed to evaluate the mortality and the variables having a P-value of less than 0.1 were included in the model to

identify independent variables by multivariate analysis. A P value of <0.05 was considered as statistically significant.

Results

A total of 185 patients were included in the study. The median follow-up period was 3.1 years. The characteristics of the patients are summarized in Table I. In terms of the development of cirrhosis complications, the 1st year, 3rd year and the 5th year follow-up examinations were performed (Table II). Ascites, variceal bleeding, hepatic encephalopathy and HCC development are summarized in Figure 1. The median duration of ascites development was 8.99 years. Survival rate was 94.6% at the 1st year, 71.9% at the 3rd year and 57.0% at the 5th year. Median survival duration was 5.83 years (Figure 2). In multivariate Cox analysis, age and serum creatinine level were found to be independently associated with mortality. Table III and Table IV show the univariate and multivariate parameters associated with mortality.

Table I. Characteristics of patients diagnosed with NASH-related cirrhosis

Characteristics of patients	N=185
Age	63.9±8.9
Sex (F/M)	116/69
Total bilirubin (mg/dl)	1.4±0.9
Albumin (gr/dl)	3.6±0.6
INR	1.3±0.4
Platelet (/mm ³)	135±68
Ferritin (mg/dl)	38 (3-674)
HbA1c*	7.5±1.8
Weight	84±18
Height	161±9
BMI (kg/m ²)	32.4±7.0
Triglyceride (mg/dl)	133±93
Ascites **	29 (15.7%)
Variceal bleeding **	28 (15.2%)
Encephalopathy **	12 (6.5%)

*In patients with diabetes,**Rate at admission
INR:International normalized ratio

Table II. The complication rate of NASH-related cirrhosis.

	1. year	3. year	5. year
Ascites (%)	26.0	40.2	46.3
Variceal bleeding (%)	19.3	21.9	21.9
Encephalopathy (%)	10.4	13.2	18.0
Hepatocellular carcinoma (%)	0.7	5.4	9.7

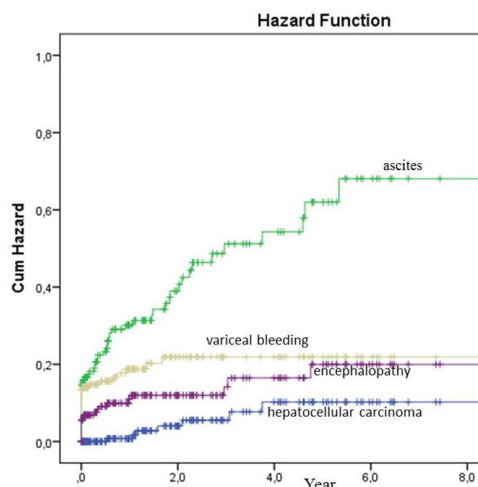


Figure 1. Complications of NASH – related cirrhosis; ascites, variceal bleeding, encephalopathy and development of hepatocellular carcinoma after diagnosis.

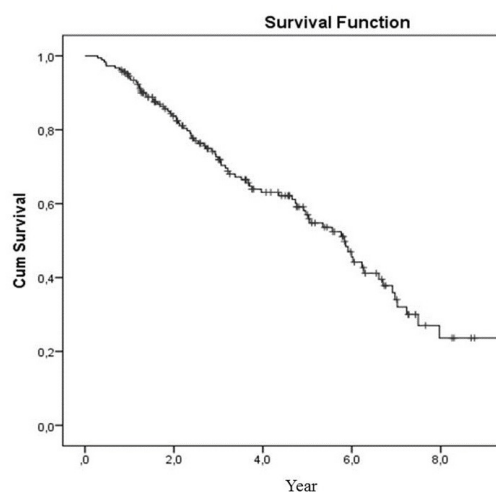


Figure 2. Mortality chart in NASH-related cirrhotic patients

Table III. Univariate Cox regression analysis for predicting mortality.

	B	Wald	P	OR	95.0% CI OR	
					Lower limit	Upper limit
Variceal bleeding*	0.162	0.36	0.549	1.176	0.692	2.000
Ascites*	0.960	12.542	<0.001	2.611	1.535	4.442
Encephalopathy*	0.979	8.841	0.003	2.662	1.396	5.076
Age	0.067	26.078	<0.001	1.07	1.042	1.097
Total bilirubin	0.214	3.536	0.06	1.239	0.991	1.549
Presence of DM	-0.044	0.007	0.932	0.957	0.347	2.637
Platelet	-0.005	5.813	0.016	0.995	0.991	0.999
Albumin	-0.918	42.081	<0.001	0.399	0.303	0.527
INR	0.700	10.056	0.002	2.014	1.307	3.104
Creatinine	2.821	7.536	0.006	16.799	2.241	125.918

*Rate at admission CI: confidence interval, OR:odds ratio

Table IV. Independent variables for predicting mortality in multivariate Cox regression analysis

	B	Wald	P	OR	95.0% CI OR	
					Lower limit	Upper limit
Age	0.116	8.564	0.003	1.123	1.039	1.214
Creatinine	3.198	7.082	0.008	24.471	2.322	257.855
Encephalopathy*	1.502	4.149	0.042	4.491	1.058	19.056

*at admission CI: confidence interval, OR:odds ratio

Discussion

The prevalence of NASH has been increasing rapidly all over the world because of sedentary life, excessive nutrition intake (primarily glucose and fructose), genetic factors, age, type 2 DM, and obesity [15]. Approximately, 11% of NASH cases progress to cirrhosis in 15 years, 7% of those who have cirrhosis progress to develop HCC in 6.5 years and 31% of cirrhotic patients proceed to decompensation in 8 years [16].

The mean age of the patients was 63.9±8.9 in this study. In a previous study, patients with NASH-related cirrhosis were reported to be elder and having more comorbidities than those with HCV cirrhosis who were on the transplantation list [17]. This difference seems to be related to the slow progression of cirrhosis due to NASH than other cirrhosis causes.

In our study, the most common complication at the end of the 5th year was ascites. The survival rate was 57.0% at the 5th year. In a study in which NASH-related cirrhotic patients were followed up for 29 months, 20% of patients had ascites, 20.8% had variceal bleeding, 20.8% had encephalopathy, and 4.2% died [18]. In our study, the data at the end of the 3rd year was similar to the results of this study.

In a study including 256 patients with NASH-related cirrhosis, any hepatic complication was observed in 19% of the patients during a mean follow-up period of 26.7 months. Survival was 92% at the 24th month, and independent predictive factors of mortality were low serum albumin level and high baseline hepatic portal venous gas (HPVG) pressure [19]. In the follow-up of 30 patients diagnosed with NASH-related cirrhosis, ascites and variceal bleeding were found to be the most common cirrhotic complications [20]. In another study, ascites was observed in 70% of patients, variceal bleeding in 24% and HCC in 9% during the 10-year follow-up period. Age, bilirubin, albumin, international normalized ratio (INR), and platelet count

parameters of the patients were reported to associate with survival independently [21]. Ascites was also the most common decompensation finding in our study.

At the time of diagnosis, 15.7% of our patients had ascites, 15.3% had hemorrhage, and 6.3% had hepatic encephalopathy. In a study evaluating patients who underwent transplantation due to NASH-related cirrhosis, 61% of the patients had ascites, 25% had encephalopathy, and 18% had variceal bleeding at the time of diagnosis [22]. In another study evaluating 354 patients diagnosed with NAFLD, 28% of the patients had portal hypertension, 33% had experienced variceal bleeding, 12% had ascites, and 7% had encephalopathy findings at the time of diagnosis [13]. Similar to these studies, the rate of decompensated cirrhosis at the time of admission was quite high in our patients.

According to our results, variceal bleeding was not independently associated with mortality which might be due to the fact that we frequently used endoscopic band ligation as a primary prophylactic measure, so other complications became more dominant on death. Besides, there was no variceal hemorrhage after the 3rd year among the patients who had no previous bleeding experience.

The cause of HCC development in NASH-related cirrhosis is the changes in apoptosis, necroptosis, and autophagy mechanisms in cells, together with increased fibrogenesis, inflammation and cellular proliferation [23]. Obesity, DM, advanced age and hepatic iron accumulation are associated with HCC development in patients with cirrhosis secondary to NASH [2, 24-26]. NAFLD is still the third most common HCC cause in the US. Considering that NASH is believed to be the most common cause of HCC in the future, NASH-related HCC has begun to attract particular attention [27]. In a United Kingdom study, there was a 10-fold increase in NAFLD-associated HCC between 2000 and 2010, and the NASH-related HCC rate was 34.8% among all HCC cases [27]. In our study, the rate of HCC at the end of the 5th year was 9.7%. In the literature, results are ranging from 9%-to-22.5% [21, 28, 29]. Previous publications have reported that NASH-associated HCC is less aggressive than HCC due to other causes [14]. In a study by Piscaglia et al., in patients with NASH-related HCC, the malignant mass was larger, more infiltrative in histological behavior and the survival was shorter than those in HCV-related HCC cases [30]. A careful and thorough examination is critical because HCC may be missed during radiological studies in these patients [14].

Cardiovascular diseases are the most common cause of death in patients with NAFLD [31]. In NASH patients with advanced fibrosis, hepatic causes are the leading cause of death [10]. Fibrosis is the most important predictor of mortality [32], while variceal bleeding due to portal hypertension is the most catastrophic complication in these patients. Variceal bleeding is responsible for 20% of NASH-related mortality [33]. In our study, the survival rate was 94.9% at the 1st year, 71.9% at the 3rd year and 57% at the 5th year. Age, serum creatinine level and presence of encephalopathy were independent predictive factors of mortality. In similar studies, mortality rates were reported as 4.2% at the end of 29 months, 8% after 24 months and 11% after 60 months [18, 19]. In these studies, low serum albumin level, high baseline HPVG pressure, patient age, bilirubin, albumin, INR, and platelet count parameters were reported to be independently associate with survival [19, 21].

The main limitations of our study were its retrospective design and the fact that some of our patients had advanced cirrhosis. In addition, we could not assess other causes of mortality such as cardiovascular mortality; we analyzed total mortality alone. Data of patient who underwent liver transplantation was not precise, too.

In conclusion, NAFLD is a complex condition associated with cardio-metabolic risk and hepatic disease. Its prevalence is increasing rapidly all over the world. In parallel, rates of cirrhosis and liver transplantation due to NASH are rapidly growing as well. NAFLD will be the most common cause of cirrhosis and liver transplantation in the near future. The development of HCC in these patients is another critical condition. Patients with cirrhosis secondary to NASH should be carefully monitored for developing HCC, varices, ascites, and hepatic encephalopathy. Patients with decompensation, such as ascites, variceal bleeding, hepatic encephalopathy, should be followed up at a transplantation capable center. Treatment aims to reduce cirrhosis progression. Lifestyle changes, such as changing the nutritional habit and doing exercise, are essential in the management. Concomitant conditions such as DM, dyslipidemia, and obesity should be managed appropriately [34]. Appropriate control of NASH would decrease the rate of development of cirrhosis and cirrhosis-related complications such as ascites, varices, HCC, and death.

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The relation between body mass index and end organ damage in white coat hypertension

Beyaz önlük hipertansiyonunda uç organ hasarı ile vücut kitle indeksi arasındaki ilişki

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ABSTRACT

Objective: White coat hypertension (WCH) is characterized by blood pressure, which is high in the outpatient clinic and normal either on ambulatory blood pressure (BP) monitoring or home BP monitoring. In this study, our objective was to investigate the effects of obesity on end organ damage and the correlation between body mass index (BMI) and end organ damage caused by WCH.

Patients and Methods: Individuals, who applied to our outpatient clinic due to other complaints or who were not diagnosed with or treated for hypertension, were enrolled in our study. Based on daytime values, systolic blood pressures below 135mmHg and diastolic blood pressures below 85mmHg were considered as WCH. The patients were examined for the findings of end organ damage. The left ventricular mass (LVM) was measured with echocardiography. Findings of hypertensive retinopathy were evaluated and albumin levels were measured.

Results: The mean left ventricular mass index (LVMI) and LVM values were $96.29 \pm 25.6 \text{ g/m}^2$ and $170.87 \pm 50.17 \text{ g}$ respectively. The rate of hypertensive retinopathy was 17%. We determined a significant correlation between BMI and LVMI independently from blood pressure levels.

Conclusion: There are conflicting conclusions about the risks related to WCH. However, several types of end organ damage can be observed independently from the blood pressure levels in this group of patients. Cardiac failure is more common and has an early onset in obese patients with WCH. In conclusion, end organ damage may emerge during the follow-up of WCH patients without a significant change in the blood pressure values.

Keywords: Hypertension, Body Mass Index, Blood Pressure.

ÖZ

Amaç: Beyaz önlük hipertansiyonu (BÖH) poliklinik ölçümlerinde kan basıncının yüksek bulunması ancak ambulatuvar kan basıncı veya ev ölçümleri sırasında kan basınçlarının normal seyretmesidir. Biz bu çalışmada, obezitenin uç organ hasarına etkisini ve vücut kitle indeksi (VKİ) ile BÖH'ün neden olduğu uç organ hasarı arasındaki ilişkiyi incelemeyi amaçladık.

Hastalar ve Yöntemler: Çalışmamıza polikliniğimize başka nedenlerle başvuran veya hastalık saptanmayan sağlıklı kişiler arasından hipertansiyon tanısı almamış ya da hipertansiyon nedeniyle tedavi edilmemiş olan bireyler dâhil edildi. Gündüz ölçüm değerleri kriter alınarak sistolik kan basıncı 135 mmHg, diastolik kan basıncı 85 mmHg'nın altındaki değerler BÖH olarak kabul edildi. Hastalar uç organ hasarı yönünden değerlendirildi. Ekokardiografi ile sol ventrikül kitlesi (SVK) ölçüldü. Retinopati araştırıldı. Mikroalbuminüri düzeyine bakıldı.

Bulgular: Ortalama sol ventrikül kitle indeksi (SVKİ) ve SVK değerleri sırasıyla $96,29 \pm 25,64 \text{ gr/m}^2$ ve $170,87 \pm 50,17 \text{ gr}$ bulundu. Hipertansif retinopati sıklığı %17 idi. Kan basıncı değerlerinden bağımsız olarak VKİ ile SVK ve SVKİ arasında korelasyon bulundu.

Sonuç: Beyaz önlük hipertansiyonunun masumiyeti konusunda çelişkili ifadeler vardır. Değişen kan basıncı değerlerinden bağımsız olarak farklı uç organ hasarları görülmektedir. Obez olan BÖH hastalarında kardiyak yetmezlik daha sık ve erken görülmektedir. Sonuç olarak BÖH'lü bir hasta izlenirken kan basınçlarında anlamlı değişiklik olmaksızın uç organ hasarı oluşabilmektedir.

Anahtar kelimeler: Hipertansiyon, Vücut Kitle İndeksi, Kan basıncı

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Introduction

Hypertension is an important health problem affecting primarily the adult population. Some authors suggest that it will have a greater impact on public health in the future [1]. In hypertensive patients, diagnosis of the end organ damage is critical in respect of the treatment choice. During the decisions on the treatment, end organ damage and concomitant diseases should be considered along with

the blood pressure levels. White coat hypertension (WCH) is characterized by a blood pressure, which is high in the outpatient clinic and normal either on ambulatory blood pressure monitoring (ABPM) or home blood pressure monitoring. Systolic blood pressure (SBP) higher than 140mmHg and diastolic blood pressure (DBP) higher than 90mmHg during the measurements in the outpatient clinic and SBP lower than 135mmHg and DBP lower than 85mmHg during ABPM is defined as WCH [2].

The prospective studies demonstrated end organ damage in WCH patients although its rate was lower than the patients with continuous hypertension [3-5]. The rate of renal involvement is also lower in WCH patients compared to the hypertensive patients but higher compared to the normotensive patients in the control group. The rates of cardiovascular events such as left ventricular hypertrophy (LVH), left ventricular diastolic dysfunction, and early-onset of the microalbuminuria are also relatively higher in WCH patients [4-6].

Like in the hypertension treatment, the goal of the treatment in WCH is the prevention of end organ damage. Therefore, the factors contributing to this process should be well defined. It is known that obesity contributes to various risk factors including cardiovascular disorders and increases the prevalence of most of the cardiovascular risk factors and induces the development of arterial hypertension [7]. Our objective in this study was to determine the effects of obesity on end organ damage and the correlation between body mass index (BMI) and end organ damage caused by WCH.

Materials and Methods

Patients

A total of 100 adult patients, who had applied to our outpatient clinic due to other complaints or healthy patients, who were not diagnosed with hypertension or treated for hypertension and had a SBP>140mmHg and DBP>90mmHg were included in the study. All included patients were older than 18 years and had no diabetes mellitus or glucose intolerance. The demographic characteristics of the patients were recorded. The height and weight of the patients were measured and the body mass index (BMI) was calculated. The study was approved by the Clinical Research Ethics Committee of Cerrahpaşa School of Medicine, Istanbul University (01.03.2011;8736).

Blood pressure measurement

The blood pressure was measured with the standardized and internationally accepted mercury manometer in the sitting position after a 20-minute resting time. The measurements were carried out in three different days. The 24-hour ABPM of the participating patients was performed with A and D Engineering TM-2421 device. During the ABPM, the daytime blood pressure levels were measured between 06:00-00:00 in every 15 minutes and the nighttime levels were measured between 00:00-06:00 in every 30 minutes and the measured values were recorded. Patients, who were diagnosed with hypertension during the outpatient examination but had a mean daytimes levels of SBP and DBP lower than 135mmHg and 85mmHg respectively were diagnosed with WCH. Patients, who had SBP and DBP levels higher than 135mmHg and 85mmHg in the daytime measurements, were diagnosed with hypertension [8].

Determination of end organ damage

The patients were examined for the end organ damage in the outpatient clinics of the cardiology and ophthalmology departments. The hypertensive changes in the retina, which were determined during the fundoscopic examination, were evaluated according to Keith, Wagener, and Barker classification [9]. The echocardiographic examination was based on the recommendations of the American Society of Echocardiography [10]. The examination was performed with M-mode two-dimension Doppler echocardiography. Hawlett Packard 2500 ultrasound device with a 2.5MHz transducer was used. The left ventricular mass (LVM) was calculated with Devereux formulation. The sections of the left ventricle were done at the junction of the mitral valve [11]. The 24-hour urine analysis was also performed. The microalbuminuria and creatinine clearance was examined in the 24-hour urine. An albumin excretion between 30-300mg/day was accepted as microalbuminuria.

Statistical Analysis

The data were expressed with mean±standard deviation. The correlation between the parameters related to BMI and end organ damage was examined with Pearson's correlation analysis. P<0.05 was considered as statistically significant. Calculations were done with SPSS v22.0 (SPSS Inc. USA) software package.

Results

Fifty female and 50 male WCH patients were included in our study (n=100). The mean age of the patient group was 48.22 years and the mean ages of female and male patients were 49±17 and 48.6±10.8 years respectively. The difference between the mean ages was not significant.

During the examination in the outpatient clinic, the mean SBP and DBP of the participating patients were 153±5mmHg and 99±25mmHg respectively. Regarding the ABPM, the mean daytime SBP and DBP levels were 121±6mmHg and 74.6±5.5mmHg; the mean nighttime SBP and DBP levels were 108.8±9.8mmHg and 65.8±7.7mmHg respectively (Table I). There was no significant correlation between the blood pressure and end organ damage.

Table I. Results of blood pressure measurements performed in the outpatient clinic and ambulatory conditions (n=100)

	Systolic blood pressure	Diastolic blood pressure
Outpatient clinic (mmHg)	>153±15	>99±25
Ambulatory (daytime) (mmHg)	121±6.4	74.6±5.5
Ambulatory (nighttime) (mmHg)	108.8±8	65.8±7.7

The mean values of BMI, LVMI and LVM were 28.80±4.33 kg/m², 96.29±25.64 g/m² and 170.87±50.17g respectively. The mean protein excretion was 28.88±44.14g/dL (Table II).

Table II. Demographic findings and parameters of the end organ damage (n=100)

	Mean±SD*
Age (year)	48.70 ± 12.44
Weight (kg)	74.66 ± 11.99
Height (m)	1.61 ± 0.07
BMI (kg/m ²)	28.80 ± 4.33
MAU (g/dL)	28.88 ± 44.14
LVM (g)	170.87 ± 50.16
LVMI (g/m ²)	96.28 ± 25.63

* Standard deviation

BMI: body mass index, MAU: Microalbuminuria, LVM: Left ventricular mass, LVMI: Left ventricular mass index

The rate of the hypertensive retinopathy (HTR) was 17% (grade I: 12%, grade II: 4%, grade III: 1%) (Table III).

Table III. The rate of the hypertensive retinopathy grades (n=100)

Grades of hypertensive retinopathy	n (%)
Grade 1	12 (12%)
Grade 2	4 (4%)
Grade 3	1 (1%)

There was a significant correlation between BMI and LVM and LVMI (P<0.05, r=0.27 and P<0.001, r=0.39 respectively). However, we did not detect any significant correlation between BMI and microalbuminuria and hypertensive retinopathy (HTR) (P> 0.05, r=0.01 and P>0.05, r=0.16 respectively) (Table IV).

Table IV. Correlation rates of the end organ damage with body mass index (BMI) (n=100)

	r	P
BMI-LVM	0.27	< 0.05
BMI-LVMI	0.39	< 0.001
BMI-MAU	0.01	> 0.05
BMI-HTR	0.16	> 0.05

BMI: Body mass index, LVM: Left ventricular mass, LVMI: Left ventricular mass index, MAU: Microalbuminuria, HTR: Hypertensive retinopathy

Discussion

Regarding the literature, there are conflicting results about the end organ damage in WCH patients. Therefore, it was suggested that the presence of various concomitant factor(s) should be responsible for these discrepancies. Nevertheless, the studies were mostly focused on the confirmation or the refusal of the presence of end organ damage development and the correlation analysis between the possible factors remained rather in the background. In this study, we investigated the role of obesity to end organ damage. The correlation of BMI and end organ damages such as retinopathy, left ventricular hypertrophy, and microalbuminuria was evaluated.

Conflicting results were also reported about the development of left ventricular hypertrophy in WCH patients. Kristensen et al., reported that LVM was lower in WCH compared to hypertension but higher compared to normotension [12]. Verdecchia et al., demonstrated that LVMI was increased in WCH compared to the normotensive subjects and was in correlation with the changing blood pressure levels. However, they did not evaluate any correlation between BMI and LVMI [13]. Owens and colleagues showed that LVM and LVMI were increased in individuals with the same blood pressure and stated that this increase in WCH occurred independently from the blood pressure levels. They did not evaluate any correlation with BMI [14]. In our study, we determined a significant correlation between BMI and LVM and LVMI.

Microalbuminuria, which is an indicator of the renal involvement, is evaluated in the hypertensive

patients. Martinez and colleagues conducted studies on hypertensive patients and showed that hypertension causes microalbuminuria and had a correlation with BMI [15]. Di Mauro and Kristensen conducted studies on WCH patients but did not detect any significant microalbuminuria in the patients [16, 17]. Palatini et al., compared WCH patients with hypertensive patients and showed that the protein excretion was increased in WCH patients but it was more prominent along with end organ damage in hypertensive patients [18]. In our study, the protein excretion was within normal limits and had no significant correlation with BMI.

In WCH patients, similar to end organ damages there was also hypertensive changes in the retina. However, its rate and severity were less prominent than the hypertensive patients. In the study of van Leiden et al, the development of retinopathy was demonstrated in hypertensive patients. In this study, the investigators detected a significant correlation between retinopathy and BMI [19]. In the study conducted by Klein and Wong, it was reported that hypertension caused retinopathy but this causality was independent from BMI [20]. In the study of Pose-Reino and colleagues, the rates of retinopathy were 58.3% and 33% in hypertension and WCH groups, respectively [21]. The same rate was 29% in the study of Cerasola et al. [22]. In our study, the rates of grade I, II and III HTR were 12%, 4% and 1% respectively in patients diagnosed with WCH (Total rate of retinopathy=17%). We observed that there was no significant correlation between retinopathy and BMI. In contrary, Pose-Reino et al. reported a significant correlation between retinopathy and BMI [21]. The presence of this significant correlation may depend on relatively prolonged exposure to WCH due to the higher mean age of the subjects and increased weight gain with the age. This increased BMI may cause a misleading correlation between BMI and HTR.

Considering the end organ damage, the conflicting results in the previous studies might depend on that the duration of the exposure to WCH was not taken into consideration during the formation of the groups. If the exposure time is not adjusted, the comparison of the groups for the end organ damage is rather difficult. The different definitions of the WCH and different age groups may be also causing these conflicting results. Therefore, instead of investigating end organ damage in the patient groups with WCH, hypertension and normotension with the absolute numeric values, we preferred to evaluate obesity, which might be effective on the development of the end organ damage, and found out that it had a strong correlation with an increase in LVM.

The “white coat effect”, which was caused by increased sympathetic activity, leads to the development of WCH. This mechanism may also explain the increase in the LVM. On the other hand, obesity shows that there is an interaction between the sympathetic activity and the satiety in central nervous system. The neurohormonal mechanisms shaping this interaction are areas that need to be investigated in the future.

There are conflicting suggestions about the risks of WCH. In WCH, various end organ damages may be encountered independently from blood pressure levels. This finding points to the presence of other factors affecting the prognosis. BMI is one of these factors causing an increase in LVM. In obese patients with WCH, the rate of cardiac failure is relatively higher and it develops in an earlier stage.

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Mass-forming extramedullary hematopoiesis mimicking Hodgkin's lymphoma

Kitle oluşturarak Hodgkin lenfomayı taklit eden ekstramedüller hematopoez olgusu

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ABSTRACT

Extramedullary hematopoiesis (EMH) refers to the proliferation of hematopoietic precursors outside the bone marrow. EMH often presents as a mass lesion in several areas of the body. In this report, we present a case misdiagnosed and explain the cause of the diagnostic error.

Keywords: Extramedullary hematopoiesis, Posterior mediastinal mass, Hemolytic anemia

ÖZ

Ekstramedüller hematopoez (EMH), hematopoetik öncül hücrelerin kemik iliği dışında çoğalması olarak tanımlanmaktadır. EMH, vücudun farklı bölgelerinde kitlesel lezyon olarak karşımıza çıkabilmektedir. Bu olgu sunumunda; ayırıcı tanı, bir tanı hatası ve olası tuzaklar üzerinden irdelenmektedir.

Anahtar kelimeler: Ekstramedüller hematopoez, Arka mediasten kitlesi, Hemolitik anemi

Introduction

Extramedullary hematopoiesis (EMH) is the proliferation of hematopoietic precursors outside the bone marrow. EMH usually presents as a mass lesion in several parts of the body such as the spleen, liver, lymph nodes and paravertebral regions among others [1]. EMH is usually asymptomatic; however, a thorough differential diagnosis is crucial to differentiate EMH from other diseases. Herein, we report a case of EMH initially misdiagnosed as a neoplasm.

Case Report

A 38-year-old male patient was admitted to the emergency room with acute chest pain. He had a history of hemolytic anemia for the past 18 years but had been noncompliant with clinical follow-up. Two months ago, he had a pulmonary hematoma due to thoracic trauma. His brother suffers from hereditary spherocytosis.

His body temperature, heart rate and blood pressure were normal. Splenomegaly was detected during palpation. Laboratory analysis results were as follows: white blood cells (WBC) 4.100/uL, hemoglobin 9g/dl, platelet 160.000/uL, AST 108U/L, ALT 95U/L, LDH 607U/L, total bilirubin 7.25 mg/dL, direct bilirubin 1.12 mg/dL and uric acid 8.56

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mg/dL. Direct and indirect Coombs tests and hepatitis serologic tests were negative. A peripheral blood smear showed normal thrombocytes, hypochromia, anisocytosis, polychromasia and 5–6 target cells in each area. In hepatobiliary ultrasonography, longitudinal length of the liver was 183 mm, whereas the spleen was measured 259 mm. Single-photon emission computed tomography (SPECT) showed a 10 ×8 cm hypodense mass lesion neighbouring the upper pole of the left kidney and extending into the posterior part of the left hemithorax and splenomegaly (Figure 1). Excisional biopsy of the mass lesion had been diagnosed as Hodgkin's lymphoma, classical type, mixed cellular subtype – in an outside hospital.

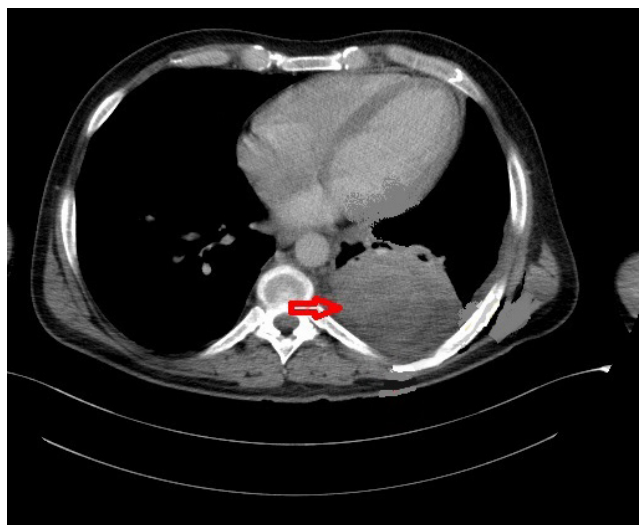


Figure 1. Radiologic image of the mass lesion (red arrow).

Finally, the patient was referred to our Hematology and Oncology Department. Hemoglobin levels declined fast and the spleen was increasing in size progressively. Hemoglobin electrophoresis, osmotic fragility, glucose-6 phosphate dehydrogenase and pyruvate kinase test results were normal. No paroxysmal nocturnal hemoglobinuria clone was detected. Positron emission tomography-computed tomography (PET-CT) scan showed minimal fluoro-deoxyglucose uptake in the mass lesion, liver and spleen. The clinical findings were linked to hypersplenism; therefore, total splenectomy and revision of the initial biopsy were planned. The paraffin block consultation of the initial biopsy was provided.

Histopathological Findings

Microscopic examination of the mass lesion showed diffuse infiltration of hematopoietic cells composed of maturing

myeloid cells, clusters of erythroid precursors and scattered megakaryocytes (Figure 2). Immunohistochemical staining confirmed the myeloperoxidase (MPO) expressing myeloid elements and glycophorin-expressing erythroid elements of the infiltrate (Figure 2). CD34 staining highlighted the background vasculature and there was no evidence of increased numbers of CD34-positive blasts. CD61 confirmed the existence of scattered megakaryocytes (Figure 2). In our repetition of immunohistochemistry, CD15 antibody stained some granulocytic lineage cells, without any Reed-Sternberg cells detected. Based on these findings the new diagnosis, in the light of the foregoing findings, was mass-forming extramedullary hematopoiesis. Subsequent splenectomy specimen exhibited histological findings of congestion, focal ischemic necrosis and old hemorrhage. There was no EMH in the spleen analysed.

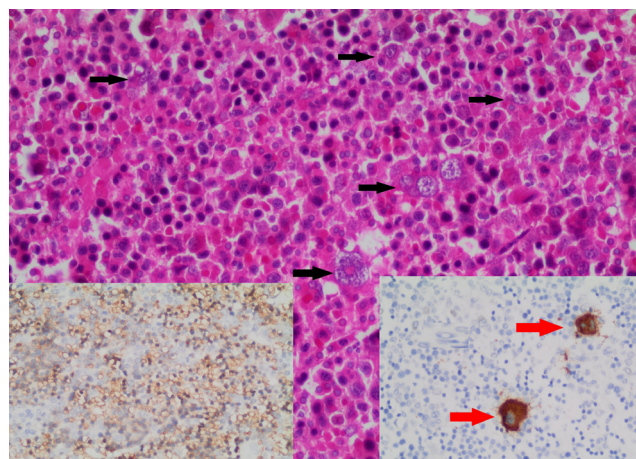


Figure 2. Mainframe: histologic section of the mass lesion (black arrows: scattered megakaryocytes, ×400). Left lower frame: glycophorin immunohistochemistry (×400). Right lower frame: CD61 immunohistochemistry (red arrows: scattered megakaryocytes, ×400).

Discussion

Extramedullary hematopoiesis occurs in several situations, such as embryonic development, insufficient bone marrow function, ineffective hematopoiesis, hypoxia, hematological disorders, and stromal disorders of the bone. EMH is characterized by hematopoietic cell proliferation in organs such as the spleen, liver, lymph nodes and paravertebral regions [1]. Rare locations previously reported are the posterior mediastinum [2], pleura [3], pericardium [4], adrenal gland [5], prostate [6] and even a pilomatricoma [7]. EMH is usually asymptomatic and develops slowly over time, but spinal cord compression [8] and spontaneous rupture [9] have been described. The most common

symptom is local vertebral pain that may be accompanied by radicular pain and paresthesia. Diagnosis of a mass-forming intrathoracic EMH can be suspected after a chest X-Ray, CT, or magnetic resonance imaging (MRI) [10]. Thoracic paraspinal masses of EMH are typically bilateral, smooth-surfaced, soft-tissue masses that contain areas of fat attenuation and do not calcify [11]. The presence of fat attenuation within the masses most likely represents non-active lesions (similar to yellow marrow), whereas enhancement is more likely to be present in actively hematopoietic masses (similar to red marrow) [12].

A posterior mediastinal mass lesion should always include a broad list of differential diagnosis including neurogenic and mesenchymal tumours, hematologic malignancies and infectious etiologies. Given the morphological findings of a mixture of hematopoietic elements; lymphomas and granulocytic sarcoma are the cardinal differentials in this case. Neither the symptoms nor the laboratory findings of the patient support a neoplastic situation. Furthermore, polyclonality of lymphoid cells is obvious and blastic cells are not present. The existence of hemolytic anemia should be reminiscent of dyshematopoiesis.

The source of confusion, in this case, was the non-specific CD15 staining in some of the megakaryocytes. CD15 (3-fucosyl-N-acetyl-lactosamine) is a cluster of differentiation antigen, also a carbohydrate adhesion molecule that can be expressed on glycoproteins, glycolipids and proteoglycans of granulocytic cells [13]. Several studies [14-16], have shown that CD15 immunoreactivity is found in a wide range of normal tissues and non-lymphoid neoplasms. This may reflect cross-reactivity with related epitopes or expression of the same epitope in a variety of tissues [17]. Although, having distinct cytological features like multilobate-vesicular nucleus, megakaryocytes may lead to the impression of Reed-Sternberg like large atypical cells, especially in a background of a mixed hematopoietic population. Clusters of erythroid precursors can be awakening, despite their lymphocyte-like appearance.

Extramedullary hematopoiesis is a non-neoplastic entity, often presenting as a mass lesion. Clinical history-especially a background of a hematologic disorder background, physical examination and radiologic findings should be included in the assessment. A multidisciplinary approach is key in this type of cases, preventing a misdiagnosis.

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