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The evaluation of sperm DNA damage in patients with different varicocele grades

Farklı varikosel dereceli hastalarda sperm DNA hasarının değerlendirilmesi

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ABSTRACT

Aim: Varicocele, abnormal dilatation of pampiniform venous plexus, is classified into three groups: 1st, 2nd and 3rd grade. The aim of our research is to show the differences among the three different varicocele grades based on the results of their sperm DNA damage and blood biochemical parameters.
Methods: We examined 30 patients which were classified into three groups: Group 1 (healthy), Group 2 (grades 1 and 2) and Group 3 (grade 3). The semen samples were examined in terms of DNA damage via comet assay. The blood samples were assessed using catalase (CAT), superoxide dismutase (SOD) enzyme activities and malondialdehyde (MDA) levels.
Results: According to the comet findings, Group 2 and Group 3 parameters were significantly higher than Group 1 ($p < 0.01$). In the biochemical findings, we observed decreased CAT and SOD activities and an increased MDA level for Group 2 and Group 3. In our research, we showed that grades 1 and 2 had significant DNA damage in terms of infertility as much as grade 3.
Conclusion: The results we derived indicate that the detection of DNA damage could be used as a predictor of infertility alongside routine semen and morphological analysis.

Key words: Varicocele, infertility, spermatozoa, DNA damage.

ÖZ

Amaç: Pampiniform venöz pleksusun anormal dilatasyonu olan varikosel üç gruba ayrılır: 1., 2. ve 3. derece. Araştırmamızın amacı, sperm DNA hasarı sonuçlarına ve kan biyokimyasal parametrelerine göre üç farklı varikosel derecesi arasındaki farklılıkları göstermektir.
Method: Grup 1 (sağlıklı), grup 2 (1. ve 2. derece) ve 3. grup (3. derece) olmak üzere üç gruba ayrılan 30 hastayı inceledik. Semen örnekleri comet testi ile DNA hasarı açısından incelendi. Kan örnekleri katalaz (CAT), süperoksit dismutaz (SOD) enzim aktiviteleri ve malondialdehit (MDA) seviyeleri kullanılarak değerlendirildi.
Bulgular: Comet bulgularına göre grup 2 ve grup 3 parametreleri grup 1'e göre anlamlı derecede yüksekti ($p < 0.01$). Biyokimyasal bulgularda CAT ve SOD aktivitelerinin azaldığını ve grup 2 ve grup 3 için MDA düzeyinin arttığını gözlemledik. Araştırmamızda 1. ve 2. derece varikoselin infertilite açısından 3. derece kadar önemli DNA hasarına sahip olduğunu gösterdik.
Sonuç: Elde ettiğimiz sonuçlar, DNA hasarının saptanmasının, rutin semen ve morfolojik analizin yanı sıra infertilitenin bir prediktörü olarak kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Varikosel, İnfertilite, spermatozoa, DNA hasarı

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INTRODUCTION

Infertility has been defined as non-conception after one year in 20-25% of couples despite regular unprotected sexual intercourse in the absence of known reproductive pathology [1]. This rate decreases to 10–15% by the end of second year. Infertility is an important concern that can affect the psychological health and social life of couples. Male factor infertility has been regarded as a contributing factor causing infertility in 45-50% percent of cases, and as the sole cause for infertility in 15-20% percent of cases [1,2]. With regard to male infertility, it is believed to be the cause of up to 35% of primary infertility and 69-81% of secondary infertility [3]. In other studies in the literature, it is understood that varicocele is one of the most frequent reasons for male infertility, excluding the idiopathic ones [4].

The effect of varicocele on spermatogenesis in sub-fertile males is related to the low amount of sperm, decrease in sperm activity and abnormal sperm morphology [4,5]. The influence of varicocele on semen parameters and infertility is explained by many pathophysiologic mechanisms: testicular temperature increase, high venous pressure, hormonal dysfunctions, autoimmunity, epididymal dysfunction, acrosome reaction disorders, renal-adrenal reflux, DNA damage and oxidative stress [5]. DNA damage is one of the most researched mechanisms in the relationship between spermatogenesis and varicocele. Many studies have confirmed a high rate of sperm DNA damage in infertile males with varicocele [6,7]. Varicocele typically damages DNA through two mechanisms. First, varicocele increases DNA fragmentation by triggering mitochondrial inactivation. This increases sperm cell apoptosis by decreasing the level of soluble FAS gene that regulates the apoptosis [8,9]. The second one is oxidative stress which is the most studied mechanism. Varicocele causes a decline in DNA polymerase activity, which repairs DNA damage. High free oxygen radical levels can lead to more chromosomal breakages, an increase in DNA fragmentation and, therefore, disorders in acrosome unity towards infertility [10-13].

While researching varicocele cases, it is critical to perform semen analyses together with physical examinations after taking the medical and reproductive background history of patients. With respect to physical examination findings, varicocele can be classified into three groups: grade 1, grade 2 and grade 3 [14]. Varicocele is the most common cause of infertility, and it can be treated through surgery that usually recommended for grade 3 patients. Operation decision is a situation that varies according to the patient. Supportive therapies can be recommended in the patient group who have been diagnosed with varicocele but have no infertility problem or whose impairment in sperm parameters is limited [15].

The purpose of our study is to investigate sperm DNA damage by utilizing the comet assay in spermatozoa samples and to calculate the oxidative stress levels by determining MDA levels and SOD and CAT activities with blood samples in different varicocele grades. Actually, we aimed to show that varicocele grade 1 and 2 had significant DNA damage in terms of infertility as much as varicocele grade 3.

MATERIALS AND METHODS

Ethic Statement and Patients

This study included the sperm samples of 10 healthy males (control group) and 20 patients who applied to Erciyes University's Faculty of Medicine, Department of Urology, with complaints of inguinal pain, infertility and distension in the testicles, and who were therefore diagnosed with varicocele. The study sample was separated into three groups. Group 1 were determined to be healthy males (n=10), group 2 were varicocele grades 1 and 2 (n=10), and group 3 were varicocele grade 3 (n=10). Semen samples were taken from patients following a 3-day sexual abstinence. We used sterile and wide-mouthed plastic containers. Samples were stored in an incubator at 37°C to be liquefied. In addition, the blood samples taken from the same patients for biochemical analyses were stored at -80°C. Also, all procedure and protocols were approved by clinical research ethics committee at the University of Erciyes (number:2013/196).

Determination of Sperm DNA Damage Using The Comet Assay

Diluted sperm samples obtained from patients were centrifuged at 300 g for 10 min at 4 °C. The supernatant was removed and the remaining sperm cells were washed with phosphate buffered saline (PBS). Damaged sperms were determined using single cell gel electrophoresis (SCGE) method called comet assay under high alkaline conditions. The images of one hundred randomly chosen cell images from the sperm sample of each patient were visually analyzed and sperm with fragmented DNA were counted. All images were recorded by using a fluorescent microscope (Olympus, BX51, Japan) through 100X zoom. The damage was determined by calculating migrated heads and broken DNA tail forming a comet. The cell with the tail was defined as damaged and the one without the tail as undamaged.

Enzyme Activities Assay: Blood samples derived from all cases in EDTA tubes were centrifuged and kept at -80°C for biochemical analyses. All analyses were done in Erciyes University, Faculty of Medicine, Department of Biochemistry.

Malondialdehyde (MDA) Assay: Standards were prepared as stated in CAYMAN Tbars Assay kit protocol. Plasmas of blood samples were taken into glass tubes. In addition, 8 glass tubes were prepared for standards. Each tube was vortex plated by adding thiobarbituric acid-sodium dodecyl sulphate (TBA-SDS) solution after filling in 100 µl of either sample or a standard. After adding 4 ml of colour reactive, tubes were left in boiling water for 1 hour. Following this period, tubes were incubated in ice for 10 min to stop the reaction. By the end of incubation the tubes were centrifuged for 10 min at 1600 G at +4°C and add 150 µl to each well of the 96-plate. Absorbance tests at 540 nm wavelength were performed and recorded.

Superoxide Dismutase (SOD) Activity Assay: Standards were prepared in accordance with protocols given with CAYMAN Superoxide Dismutase kit. Each well was filled with 10 µl of sample or standard, 200 µl diluted radical detector and, finally, 20 µl diluted Xanthine Oxydase (KO), and the reaction was started to be incubated for 20 minutes in the shaker at room temperature. Absorbance tests at 460 nm wavelength were performed with plate reader and recorded.

Catalase (CAT) Activity Assay: Standards were prepared in accordance with protocols given with CAYMAN Catalase kit. Each well was filled with 20 µl sample, 30 µl methanol and 100 µl diluted assay buffer. The reaction was started with the addition of hydrogen peroxide (H2O2) to all wells. Wells were incubated for 20 minutes in the shaker at room temperature. In order to stop the reaction, 30 µl potassium hydroxide (KOH) was added and left for 10 minutes for incubation at room temperature following the addition of 30 µl catalase purpald into each well. Then, 10 µl catalase potassium periodate was added for incubation in shaker for 5 minutes at room temperature. Absorbance tests at 540 nm wavelength were performed with plate reader and recorded.

Statistical Analyses: The Shapiro-Wilks test was used to identify normal distribution of the data. Significant difference between two treatment groups was performed using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey Test. P values less than 0.05 level were accepted as statistically significant.

RESULTS

Comet Assay Technique

In the current study, the alkaline comet technique was used to determine the single and double helix denaturations in sperm cell DNA of varicocele cases. Head length (length head), tail length (length tail), comet length (length comet), head % DNA (head DNA) and tail % DNA (tail DNA) parameters were analysed using Comet Assay Software Project-1.2.2 (CASP). The damage was determined through the calculation of migrated and comet-caused DNA tails. The extent of damage was calculated by adding parameters of tail length, fluorescence level at head and tail, and tail moment. The DNA fluorescence percentage of the tail is considered to be directly proportional to the frequency of DNA chain breakage. Comet assay results of groups are shown in Figure 1.

Head Length

It was statistically shown that the measured head lengths in Group 3 decreased compared to Group 1 and Group 2 ($p < 0.05$), however, no significant difference was observed between Group 1 and

Group 2 (p > 0.05). Results were shown in Figure 2.

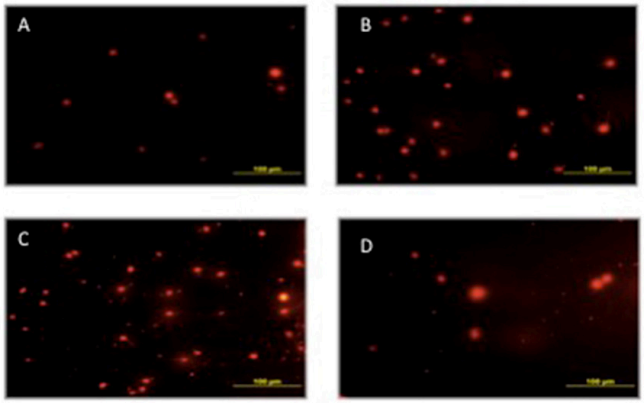

Figure 1. Intergroup sperm comet images A) Group 1 (Control Group), B) Varicocele Grade 1 1, C) Varicocele Grade 2, D) Varicocele Grade 3 (Ethidium Bromide Staining,x100)

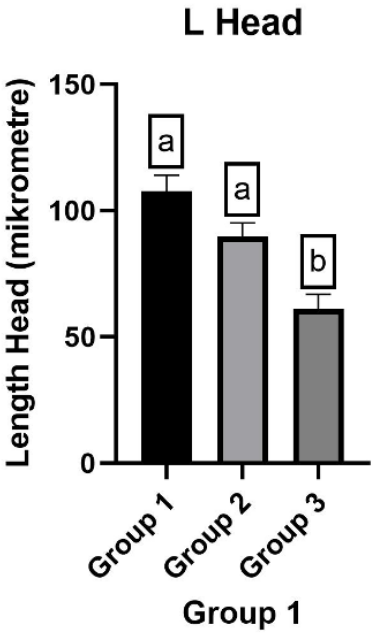

Figure 2. Statistical comparison of head length. There was a statistically significant difference between group 3 and the other groups (p < 0.05). However, no significant difference was founded between Group 1 and Group 2 (p > 0.05). While a statistical difference was observed between the groups labeled with different letters (p <005), there was no significant difference between the groups labeled with the same letter (p> 0.05).

Tail Length

The tail length measured in Group 2 and Group 3 showed a statistically significant increased compared to Group 1 (p < 0.05). Nevertheless, the difference between Group 3 and Group 2 was also statistically significant (p < 0.05). Results were shown in Figure 3.

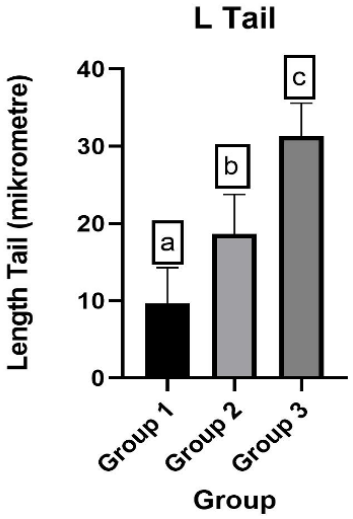

Figure 3. Statistical comparison of tail length. The tail length of Group 2 and Group 3 increased compared to Group 1 and this increased was found to be statistically significant. (p < 0.05). The most prominent increase was in Group 3 (p < 0.001). Statistical significant difference in groups was showed with different letters (p < 005).

Comet Length

A statistically significant increased was observed between Group 3 and the other groups (p < 0.05). However, no significant difference was observed between Group 1 and Group 2 (p > 0.05). Results were shown in Figure 4.

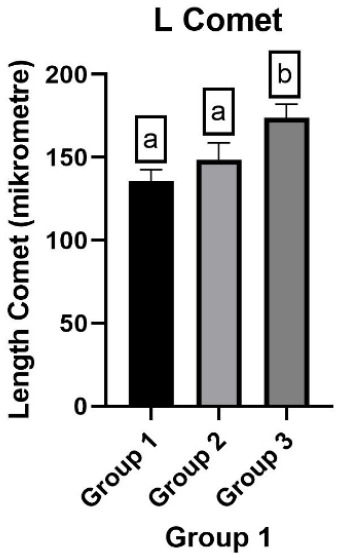

Figure 4. Statistical comparison of comet length. No significant difference was observed in comet length of Group 2 compared to Group 1 (p > 0.05). However, There was a statistically significant increased between group 3 and the other groups (p < 005). While a statistical difference was observed between the groups labeled with different letters (p <005), there was no significant difference between the groups labeled with the same letter (p> 0.05).

Percent Tail DNA

Percentage tail DNA measured in Group 3 and Group 2 increased gradually compared to Group 1 ($p < 0.05$). Looking at the findings, we can state that increase in percentage of tail DNA and varicocele grades are positively correlated to each other. Results were shown in Figure 5.

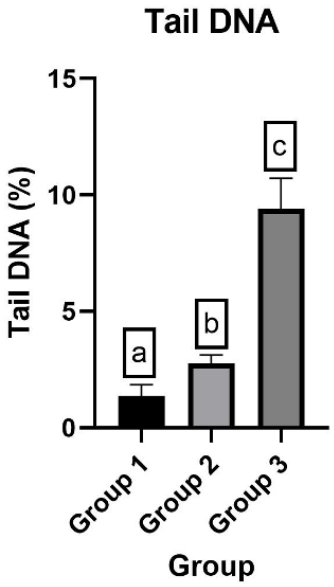

Figure 5. Statistical comparison of the percent tail DNA. Percentage tail DNA measured in Group 3 and Group 2 increased gradually compared to Group 1 ($p < 0.05$). Statistical significant difference in groups was showed with different letters ($p < 005$).

Percent Head DNA

Percentage DNA in the comet head measured in Group 2 and Group 3 decreased gradually compared to Group 1 ($p < 0.05$). There was a prominent decrease in the percent head DNA as the degree of varicocele increased among the groups ($p < 0.05$). According to the findings we can inform that decrease in percentage of head DNA and varicocele grades are positively correlated to each other. Results were shown in Figure 6.

Biochemical Results

Blood malondialdehyde (MDA) level, Catalase (CAT) and Superoxide Dismutase (SOD) enzyme activities were evaluated in blood samples by ELISA technique. CAT and SOD levels as indicators of oxidant/anti-oxidant presence and the MDA level as the indicator of lipid peroxidation were measured in the blood samples obtained from our cases. The measurement results are given in Table 1; $p < 0.010$ is accepted as statistically meaningful.

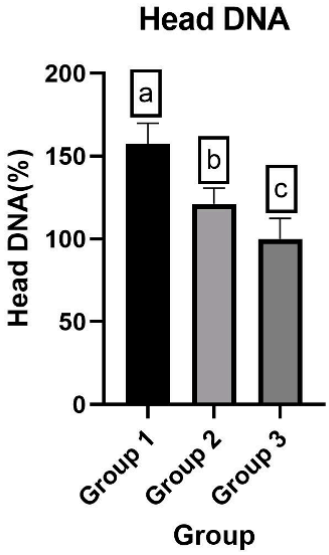

Figure 6. Statistical comparison of the percent head DNA. Percentage DNA in the comet head measured in Group 2 and Group 3 decreased gradually compared to Group 1 ($p < 0.05$). Statistical significant difference in groups was showed with different letters ($p < 005$).

Serum Malondialdehyde (MDA) Levels

MDA levels were measured in the serums of blood samples to determine the peroxidation level. MDA levels are given in Table 1. When intergroup MDA levels are compared, the MDA level of Group 3 was significantly higher than Groups 1 and 2 ($p < 0.010$). When the MDA level of Group 2 was compared to that of Group 1, it was higher, but lower than that of Group 3, and the result was statistically meaningful ($p < 0.010$).

Table 1. Statistical Comparison of Enzyme Parameters

	MDA (µM)	CAT (µM)	SOD (µM)
Group 1 (n=10)	17,64±9,67a	25,47±11,02a	0,23±0,19a
Group 2 (n=10)	75,01±32,22b	4,05±2,39b	0,06±0,09b
Group 3 (n=10)	118,96±52,10b	1,69±1,31b	0,02±0,05b
p	0,001	0,001	0,001

While a statistical difference was observed between the groups labeled with different letters ($p <005$), there was no significant difference between the groups labeled with the same letter ($p> 0.05$).

Serum Catalase (CAT) Activity

The mean CAT activities were measured in the serums of blood samples to determine the peroxidation in them and were given in Table 1. When Group 1 was compared to other groups, the

mean CAT activity was higher than that of other groups and this was statistically meaningful ($p < 0.010$). When Group 2 was compared to Groups 1 and Group 3, the activity level was lower than Group 1, and the result was statistically meaningful ($p < 0.010$); despite being higher than Group 3, it was not statistically meaningful ($p = 0.302$). However, when Group 3 was compared to Groups 1 and 2, we have observed that Group 3 was lower than Group 1 and this was statistically meaningful ($p < 0.010$) and lower than group 2 but not statistically meaningful ($p = 0.302$).

Serum Superoxide Dismutase (SOD) Activity

SOD activities were measured in the serums of blood samples to determine the peroxidation levels in them. SOD activities were given in Table 1. The mean SOD activity level of Group 1 was higher than that of the other groups, and this was statistically significant ($p < 0.010$). When Group 2 was compared to Groups 1 and Group 3, the mean SOD activity level was lower than Group 1, and the result was statistically meaningful ($p < 0.010$); despite being higher than Group 3, it was not statistically meaningful ($p = 0.530$). However, when Group 3 was compared to Groups 1 and 2, the result for Group 3 was lower than Group 1 and this was statistically prominent ($p < 0.010$). The result for Group 3 was lower than Group 2, but the result was not statistically remarkable ($p = 0.530$).

DISCUSSION

In the literature, there are many studies on varicocele and sperm DNA damage. The DNA quality in males is equivalent to reproductive ability. A DNA-damaged sperm can enable fertilization to proceed but is the subject of research due to the high possibility of aneuploidic embryos, early pregnancy losses, the risk of metabolic diseases as a result of epigenetic changes, and childhood cancers [17]. Recent studies have shown that varicocele has effects on semen parameters causing meaningful damage to sperm DNA, causing hormonal destruction by affecting the structure of Sertoli and Leydig cells in the testicles, and triggering direct oxidative damage by increasing Reactive Oxygen Species (ROS) levels [18]. In light of prior studies, in our study, we have investigated the sperm nuclear DNA damage and measured oxidative stress levels in patients with different grades of varicocele compared to normal healthy males. It is reported that sperm function may be damaged due to a dysfunctional acrosome or autoimmune reaction related to varicocele pathology [18]. Today, it is well-known that reactive oxygen radicals increasing secondarily to oxidative stress causes damage through lipid peroxidation in cells. The target of these reactive oxygen radicals is unsaturated fatty acids in the cell membrane, and they may affect any cell that has these acids. It is stated that as the sperm membrane is rich with unsaturated fatty acids, increasing reactive oxygen radicals due to varicocele pathology may also affect the sperm structure [11]. Köksal et al. [19] have demonstrated that reactive oxygen radicals with varicocele generated rats are higher in number than the normal population. In a study where left varicocele cases were examined, it has been reported that ROS was related to varicocele, and, therefore, there was an increase in DNA fragmentation [20]. Similarly, Allamaneni et al. [21] have reported that when varicocele grade 3 is compared to grades 1 and 2, seminal ROS levels were found to be meaningful. In another study conducted by Smith et al. [22] it has been stated after their research on the the mechanisms which play role in varicocele that males with varicocele had more free oxygen radical amounts in semen. In our study, we analysed ROS and antioxidant parameters in cases of different varicocele phases. Our results are in accordance with those of Allamaneni et al. [21], and we have shown that the varicocele grade was higher in parallel to higher ROS parameters, while antioxidant levels were meaningfully lower.

It is curious subject whether there is a relationship between DNA damage and parameters such as ROS in male infertility. Saleh et al. [23] have reported that damage to sperm DNA had a negative impact on fertility. The DNA damage caused by ROS accelerates cell apoptosis. This has a negative impact on reproduction biology due to low number of sperm. In many studies, it has been stated that the determination of sperm morphology was not sufficient to find the cause of infertility. Thus, many different techniques have been preferred for determining sperm DNA damage in recent past. However, some studies mention that such techniques used for the determination

of only sperm DNA damage have no superiority in morphologic evaluation [24]. We have analysed sperm DNA damage together with blood oxidative stress levels, and we have concluded that there was a positive correlation between the two. Ying-Jun Wang et al. [25] have shown in a meta-analysis of 83 independent studies on varicocele and sperm DNA damage published between 1963 and August 2011 that the best techniques for determining sperm DNA damage were terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), comet assay and sperm chromatin structure assay (SCSA). Simon et al. [26] have performed a spermatozoon analysis in three groups for DNA damage in infertile couples by using comet assay with the idea that sperm DNA damage affects embryo quality: low damage, medium damage and high damage. The reasons for the infertility of these couples were in three groups: male, female and undefined. Each embryo was categorized as good, medium or low quality; when they were compared in terms of the effect of sperm DNA damage on embryo quality, the spermatozoon group with low DNA damage was meaningfully higher with high quality embryo percentages. Therefore, we have utilized comet assay in order to show the DNA damage in different grades of varicocele in our study. comet assay is often preferred for DNA damage measurements as it is simple, fast, precise, applicable for different cell types and DNA damages, and, most importantly, it does not require any radioactive labelling [27].

Conclusion: The present study concluded that there was more sperm DNA damage in varicocele grades 1 and 2 cases than expected, and there was high DNA damage in all varicocele grade 3 cases. Our comet findings are concordant with biological parameters. This condition may imply that varicocele grade 1 and 2 are significant DNA damage in terms of infertility as much as varicocele grade 3. Therefore, the detection of DNA damage could be used as a predictor of infertility alongside routine semen and morphological analysis.

Conflict of Interest: No conflict of interest was declared by the authors.

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