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Research Article

Testicular Apoptosis and Proliferation in Relation to Body Mass Index and m-TESE Success in Nonobstructive Azoospermic Men

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Male infertility occurs due to the disruption of the balance of proliferation and apoptosis in the testicular tissue. It is important to show the effect of increased body mass index, which is one of the factors that disrupts this balance of apoptosis and proliferation, not only at the blood hormone level but also at the tissue level. For this reason, the present study is aimed at demonstrating the relation between body mass index and cell turnover in the testis using immunohistochemical methods. In the present study, patients were grouped as normal, overweight and obese, and as m-TESE positive and negative. The sperm retrieval rate with microsurgical testicular sperm extraction (m-TESE) was 41.67%. Histological diagnosis of testicular tissues was made with hematoxylin and eosin and Masson's trichrome staining. Apoptosis and proliferation in the testicular tissue were demonstrated by TUNEL and PCNA immunohistochemical methods, respectively. It was concluded that BMI had no significant effect on reproductive hormone profile (FSH, LH, and testosterone), m-TESE success, apoptosis, and proliferation in testicular tissue in nonobstructive azoospermic men. In addition TUNEL positivity and proliferative index was found to be significantly correlated with testicular histology and m-TESE outcome.

1. Introduction

Infertility is defined as the absence of spontaneous pregnancy despite regular unprotected sexual intercourse for one year [1]. Infertility is caused by 20-30% male, 20-35% female, and 30-40% both female and male factors. The rate of unexplained infertility is 10-20% [2]. Although 30-40% of male infertility is idiopathic, many factors play role in its etiology. Causes of primary spermatogenic or testicular failure are congenital factors (anorchia and testicular dysgenesis), trauma, torsion, tumor, infection, drugs, and temperature [3, 4]. This leads to nonobstructive azoospermia (NOA), and patients usually have hypergonadotropic hypogonadism. In hypergonadotropic hypogonadism, which is more common, testosterone levels are low, while FSH and LH levels are high. Secondary hypogonadism occurs as a result of congenital and acquired disorders of the hypothalamus and pituitary gland. Causes of acquired secondary hypogonadism are diabetes, morbid obesity, liver cirrhosis, and idiopathic hypogonadotropic hypogonadism. In secondary hypogonadism, FSH, LH, and testosterone levels are low [3].

Infertile male patient is evaluated by history, physical examination, hormone analysis, semen analysis, and necessary genetic analysis [5, 6]. In the treatment of male infertility, fertility-related lifestyle changes should be recommended. Environmental factors that impair sperm production and function should be avoided. Making lifestyle changes such as proper nutrition, avoiding adverse environmental conditions, and exercising contributes positively to the treatment process by eliminating environmental factors that cause infertility [7]. Azoospermia, absence of spermatozoa in a semen sample, is seen in 1% of the general population and 10-15% in infertile men [8]. Azoospermia is classified as obstructive azoospermia (OA) or nonobstructive azoospermia (NOA). NOA occurs as a result of impaired spermatogenesis. In NOA patients, m-TESE is performed for therapeutic purposes, and in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI) is performed in case mature sperm cells are found in the testicular

tissue. m-TESE was first described in 1999 [9]. The rate of sperm finding as a result of m-TESE in NOA patients is 50% [4, 10, 11]. While the sperm retrieval rate can reach 85% in hypospermatogenesis cases, this rate decreases to 6-25% in cases such as germinal aplasia [12]. In NOA, the probability of finding sperm in the testis is predicted according to age, body mass index, smoking, testicular volume, hormone (FSH, LH, testosterone, and prolactin) level, genetics, and testicular histopathology [13–16]. No factor has yet been proven to predict sperm retrieval from azoospermic patients by surgical methods, and studies are still ongoing on this subject [17].

Obesity is a medical condition in which white adipose tissue accumulates above normal in the body and affects organs and systems in a way that impairs their function. It is a multifactorial disease in which a predisposing genotype is associated with environmental factors such as sedentary lifestyle and excess calorie intake. A body mass index (BMI) of 25-30 kg/m^2 is defined as overweight, and over 30 kg/m^2 is defined as obesity [18]. In recent years, when obesity has increased rapidly, the decrease in semen quality and reproductive functions and the increase in infertility rates have attracted attention [19, 20]. In recent years, studies have focused on the issue that there may be a relationship between decreased fertility and increased obesity. Studies are ongoing to elucidate the underlying mechanisms. It is emphasized that obesity affects semen parameters by disrupting the hormone profile (low FSH, LH, testosterone, inhibin B, and high estrogen) causing an increase in scrotal temperature due to scrotal adiposity and increasing oxidative stress and inflammation in the male reproductive system [21]. In addition to low testosterone, low FSH and LH and hypogonadotropic hyperestrogenic hypoandrogenemia are observed in obese men [22]. Low testosterone and high estrogen impair normal testicular function and spermatogenesis.

Imbalance of testicular proliferation and apoptosis results in male infertility. Increased body mass index is one of the factors that disrupts this balance. The aim of this study was to associate apoptosis and proliferation on the testicular level by comparing m-TESE positive and m-TESE negative NOA patients with regards to subgroups assigned according to BMI. m-TESE outcome has been addressed before but mainly with the focus of endocrine changes, and the topic is still debated controversially. This study is aimed at analyzing the cellular turnover in testicular level with the help of PCNA and TUNEL methods.

2. Materials and Methods

2.1. Subject Selection, Data Collection, and Tissue Sampling. Approval was obtained from Marmara University Clinical Research Ethics Committee (09.2019.365). Twelve azoospermic patients who applied to Istanbul University, Istanbul Medical Faculty, Department of Andrology were included in the study. Written consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used. Height and weight of patients were measured, and body mass indexes were calculated according to WHO and recorded [23]. Most recent hormone levels (FSH, LH, and testosterone) and spermiogram values, which were analyzed by the andrology laboratory according to WHO guidelines [24], were obtained from patients' files. 20-25 kg/m^2 was defined as normal weight, 25-30 kg/m^2 as overweight, and over 30 kg/m^2 as obese. Patients without chromosomal anomaly or Y chromosome microdeletion were included in the study.

2.2. Histological Examination. Half of the testicular tissue sample obtained intraoperative from each patient was placed in Bouin's solution for histochemical methods and the other half in 10% neutral-buffered formalin solution for immunohistochemical methods and fixed for 24 hours. After fixation, the tissues were passed through the increasing ethanol series and cleared in xylene. Sections of 4 μm thickness were obtained. Hematoxylin and eosin stain was used for general morphological evaluation of the sections and Masson's trichrome stain to show interstitial and peritubular fibrosis [25]. Histological classification of testicular biopsy was made as normal spermatogenesis, hypospermatogenesis, maturation arrest, germ cell aplasia (Sertoli cell-only), and seminiferous tubule hyalinization [26]. The degree of fibrosis was evaluated semiquantitatively and ranged from 0 to 3 (0, < 5%; 1, 6–25%; 2, 26–50%; and 3, > 50%) [27]. The sections were examined and photographed by an Olympus DP72 camera-attached Olympus BX51 light microscope under ×200 and ×400 magnifications.

2.3. Immunohistochemistry

2.3.1. PCNA Immunohistochemistry. Sections of 4 μm thickness were deparaffinized and hydrated. Sections kept in 3% hydrogen peroxide solution prepared with methanol for endogenous peroxidase blockade were exposed to microwave in heated citrate buffer for antigen retrieval. They were then incubated in primary rabbit anti-PCNA antibody (dilution 1 : 100) (ab15497, Abcam, Cambridge, UK) overnight. Sections were incubated in antipolyvalent biotinylated secondary antibody (SHP125, ScyTek Laboratories, Inc., USA), streptavidin-peroxidase conjugate, and then in 3,3′-diaminobenzidine (DAB) substrate solution (DAB Chromogen/Substrate Bulk Pack, ACK125, ScyTek Laboratories, Inc., USA) by observing the reaction formation under the microscope. Counterstaining was done with Mayer's hematoxylin.

Proliferative index (PI) was calculated by counting the positive cells in 10 randomly selected seminiferous tubules from a section of each sample at 400x magnification under the microscope and dividing them by the total number of cells (PI: PCNA positive cell count/total cell count × 100%) [28]. The sections were examined and photographed with an Olympus DP72 camera-attached Olympus BX51 light microscope under ×40 objective.

2.3.2. TUNEL Method. For the detection of apoptosis, in situ DNA end labeling technique was used (ApopTag Plus Peroxidase In Situ Apoptosis Kit S7101, Merck KGaA, Darmstadt, Germany). Deparaffinized and hydrated sections were treated with proteinase K (IHC Select Proteinase K, 21627, Merck KGaA, Darmstadt, Germany). The sections were kept in 3% hydrogen peroxidase solution for endogenous

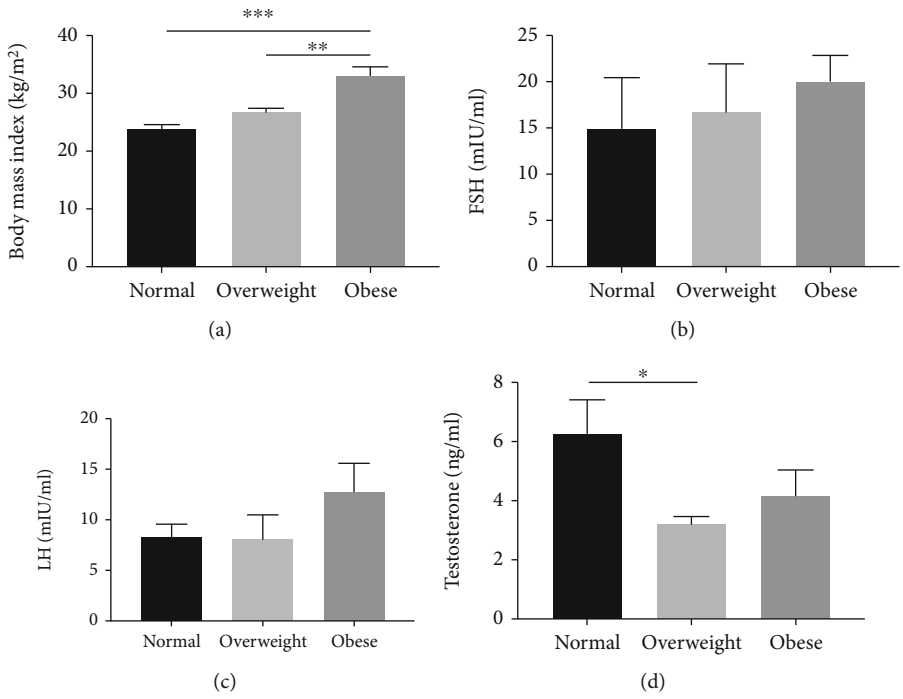

Figure 1: BMI groups (a) and their FSH (b), LH (c), and testosterone (d) levels.

peroxidase blockade. After the buffer solutions applied according to the manufacturer's guideline, the sections were stained with 3,3′-diaminobenzidine (DAB) chromogen solution until a color reaction was observed under the microscope. Counterstaining was done with Mayer's hematoxylin. Cells with the same morphology and staining characteristics as the positive control in the sections were considered TUNEL positive. For the negative control, a tissue section was incubated only with reaction buffer in the TdT enzyme step. The sections were examined and photographed by an Olympus DP72 camera-attached Olympus BX51 light microscope under ×40 objective.

2.4. Statistical Analysis. GraphPad Prism 7 and ImageJ programs were used for statistical analysis. Data were analyzed by using one-way ANOVA for BMI subgroups and unpaired t-test for m-TESE subgroups. $p < 0.05$ was considered significant.

3. Results

3.1. BMI, Reproductive Hormone, and m-TESE Findings. BMIs of the patients were grouped as normal ($n = 3$), overweight ($n = 6$), and obese ($n = 3$) (Figure 1(a)). When the three groups were compared with each other, it was determined that there was no significant difference between BMIs and serum FSH and LH levels. Serum FSH and LH levels showed a tendency to increase in obese group (Figures 1(b) and 1(c)). The testosterone level was significantly lower in the group with overweight BMI than the normal group. Although the testosterone level is also lower in the obese group compared to the normal group, there is no significant difference. This result is likely to be due to the small number of samples (Figure 1(d)).

The success rate of m-TESE was found to be 41.67%. When the patients were grouped as successful ($n = 5$) and unsuccessful ($n = 7$) m-TESE, the mean (±SEM) ages between the two groups were 37 ± 1.9 and 35.57 ± 2.6, respectively. Serum FSH levels of m-TESE positive and m-TESE negative groups were 13.54 ± 3.45 and 19.21 ± 4.12 mIU/ml (Figure 2(a)); serum LH levels were 10.09 ± 2.20 and 8.40 ± 1.90 mIU/ml (Figure 2(b)), and serum testosterone levels were 4.47 ± 0.60 and 4 ± 0.75 ng/ml (Figure 2(c)), respectively. Serum reproductive hormone levels showed no significance comparing to m-TESE success.

3.2. Light Microscopic Findings. Testicular biopsies of 12 azoospermic patients included in the present study were evaluated light microscopically by staining with hematoxylin and eosin and Masson's trichrome. One patient was diagnosed with hypospermatogenesis. In the case of hypospermatogenesis, all steps of spermatogenesis were present in the seminiferous tubules, but decreased spermatogenesis was observed (Figure 3(a)). Tissue samples of 33.3% of the patients ($n = 4$) were defined as maturation arrest. In these cases, tubules with arrested spermatogenesis and tubules with germ cell aplasia coexisted (Figure 3(b)). Fifty percent of the patients ($n = 6$) were diagnosed with germ cell aplasia (Sertoli cell-only syndrome). In these cases, tubules only lined with Sertoli cells, atrophic and hyalinized tubules, or focal and/or diffuse Leydig cell hyplerplasia in interstitial areas were observed (Figures 3(c) and 3(d)). Peritubular and interstitial fibrosis shown with Masson's trichrome stain were not observed in a case of hypospermatogenesis (Figure 3(e)). Peritubular and interstitial fibrosis were not

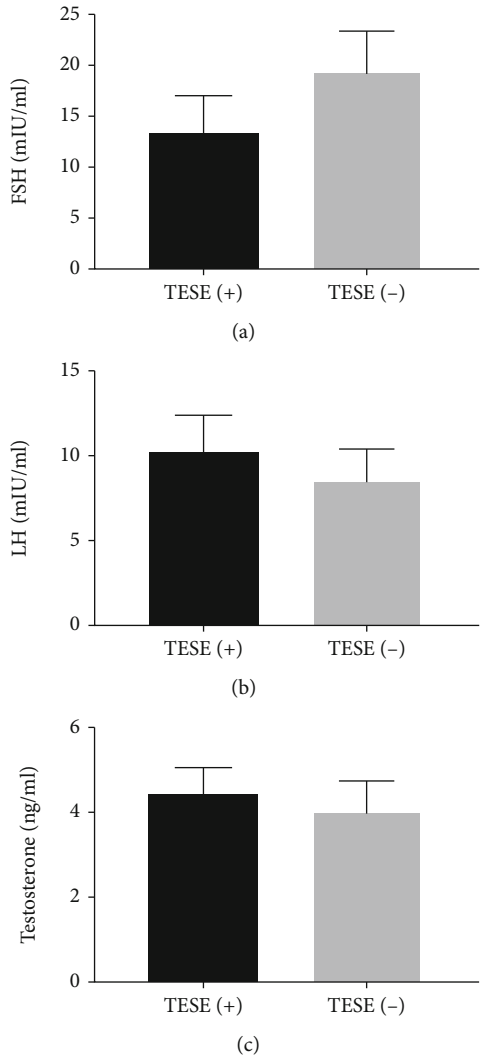

Figure 2: Serum FSH (a), LH (b), and (c) testosterone levels according to m-TESE positivity.

observed in maturation arrest cases, and an increase in fibrosis was noted only around the tubules where spermatogenesis was not observed (Figures 3(f) and 3(g)). In germ cell aplasia, both peritubular and interstitial fibrosis were present (Figure 3(h)). In one patient, there was no seminiferous tubule structure in the testicular tissue, and completely, fibrotic tissue was observed. In three patients with normal BMI, the biopsy of one patient showed hypospermatogenesis, and the other two showed germ cell aplasia. In the BMI overweight group, the biopsies of two patients showed maturation arrest, three showed germ cell aplasia, and one showed fibrosis. In the BMI obese group, two patients had maturation arrest, and one patient had germ cell aplasia. Of the five patients with positive m-TESE, one had hypospermatogenesis, and four had maturation arrest. Six of the seven patients with negative m-TESE had germ cell aplasia, and one had fibrotic tissue.

Fibrosis was graded semiquantitatively. According to m-TESE success, the degree of fibrosis was significantly higher in the negative group than in the positive group (Figure 4(a)). The degree of fibrosis did not differ significantly according to body mass index (Figure 4(b)).

3.3. PCNA Findings. When the cases were separated as m-TESE positive and m-TESE negative, the PIs (mean ± SEM) of the two groups were calculated as 0.32 ± 0.09 and 0.04 ± 0.01, respectively, and a statistically significant difference was found between the groups ($p < 0.01$) (Figure 4(c)). It was evaluated that there was a statistically significant relationship between the histological type and PI ($p < 0.01$) (Table 1). When the cases were divided into three groups as normal, overweight, and obese according to their BMIs, the PIs of the groups were calculated as 0.27 ± 0.22, 0.10 ± 0.04, and 0.17 ± 0.07, respectively, and no significant relationship was found between BMI and PI (Figure 4(d)). While the highest proliferative index was in hypospermatogenesis (Figure 5(a)), PI showed a decrease in maturation arrest (Figure 5(b)), germ cell aplasia, and fibrosis.

3.4. TUNEL Findings. TUNEL positive cells were detected in 33.3% of the patients ($n = 4$). Diagnoses of these cases were hypospermatogenesis (Figure 5(c)) and maturation arrest (Figure 5(d)). Of the cases without TUNEL positive cells, one had maturation arrest, six had germ cell aplasia, and one had fibrotic tissue. There was a significant correlation between the diagnosis and the presence of TUNEL positive cells ($p < 0.01$) (Table 2). In addition, TUNEL positive cells were detected in four of the five m-TESE successful cases, while TUNEL positive cells were not detected in any of the unsuccessful cases. The relationship between TUNEL positivity and the presence of sperm in m-TESE was found to be statistically significant ($p < 0.01$) (Table 3). When the cases were divided as normal, overweight, and obese according to their BMIs, there was no significant difference between the groups.

4. Discussion

The importance and purpose of apoptosis in testis are to eliminate genetically defective cells that may occur as a result of mitosis and meiosis and thus to ensure that germ cells are of the required number and quality [29]. Disruption of the balance between proliferation and apoptosis leads to impaired spermatogenesis and results in infertility. Increased apoptosis in the case of abnormal spermatogenesis resulting in male infertility has been demonstrated in human studies [30]. However, the underlying causes and molecular mechanisms continue to be elucidated.

In studies on male infertility scanned in the literature, it was stated that positivity in Sertoli cells is mostly not detected as a result of the TUNEL method [31]. This suggests that Sertoli cells are resistant to apoptosis. Studies have shown that Sertoli cells are rich in proapoptotic factors compared to germ cells; therefore, they are resistant [32]. In the present study, in accordance with the literature, Sertoli cells were found to be resistant to apoptosis. Germ cell aplasia was diagnosed by routine staining techniques in half of the patients. The absence of TUNEL positive cells in specimens with germ cell aplasia reveals the resistance of Sertoli cells to

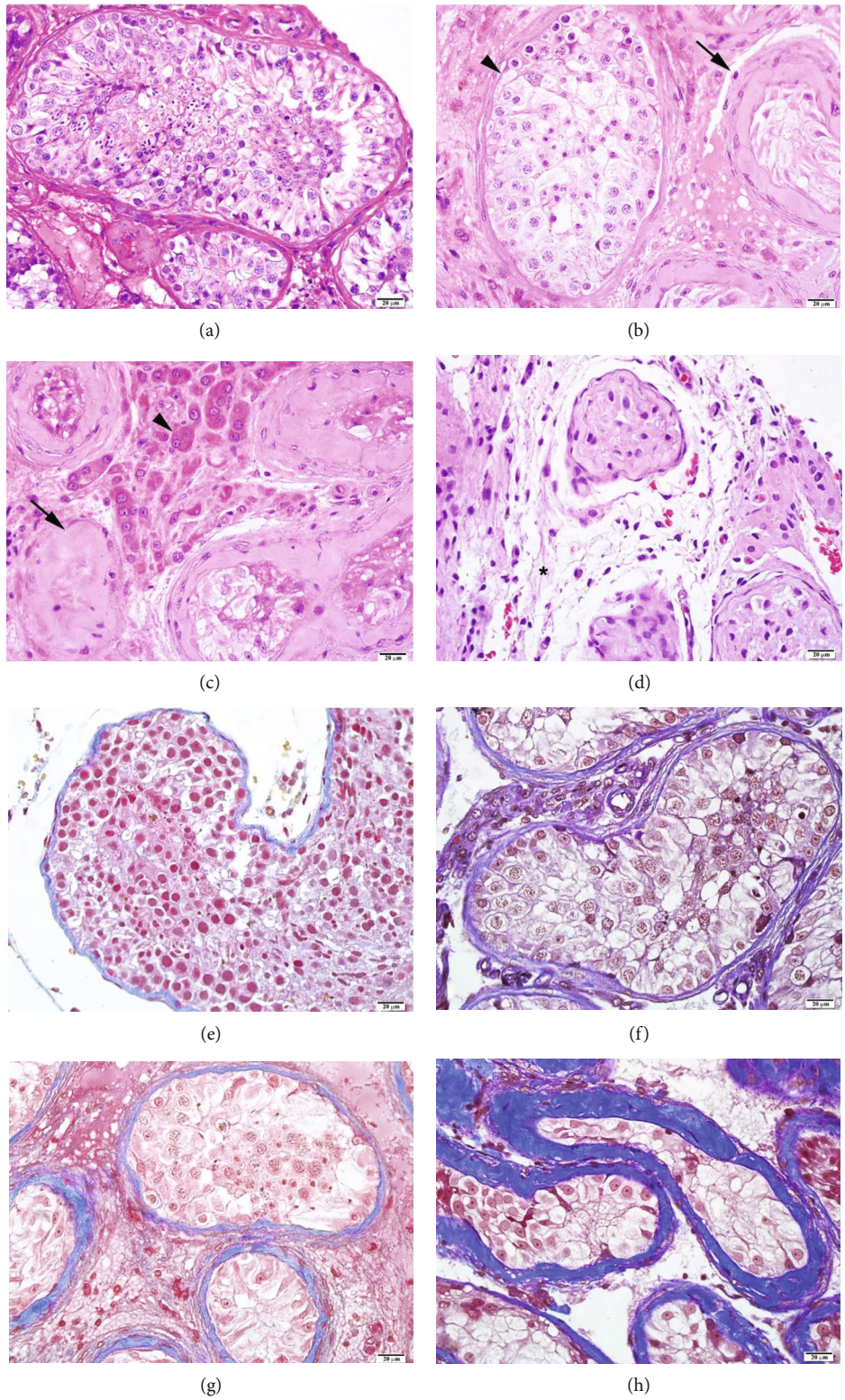

Figure 3: (a) Hypospermatogenesis. (b) Heterogeneous maturation arrest. Tubule containing maturation arrest in the spermatid stage (arrowhead) and tubule containing only Sertoli cells in which hyalinization has started (arrow) are observed together. (c) Germ cell aplasia. Leydig cell hyperplasia with round nuclei with eosinophilic cytoplasm in interstitial tissue (arrowhead) and hyalinized tubule (arrow). (d) Germ cell aplasia with interstitial fibrosis (asterisk). (e) Grade 0 fibrosis (hypospermatogenesis). (f) Grade 1 fibrosis (maturation arrest). (g) Grade 2 fibrosis (seminiferous tubule with maturation arrest and germ cell aplasia together). (h) Grade 3 peritubular and interstitial fibrosis in a case of germ cell aplasia. (a–d) Hematoxylin and eosin staining. (e–h) Masson's trichrome staining. Bars: 20 μm.

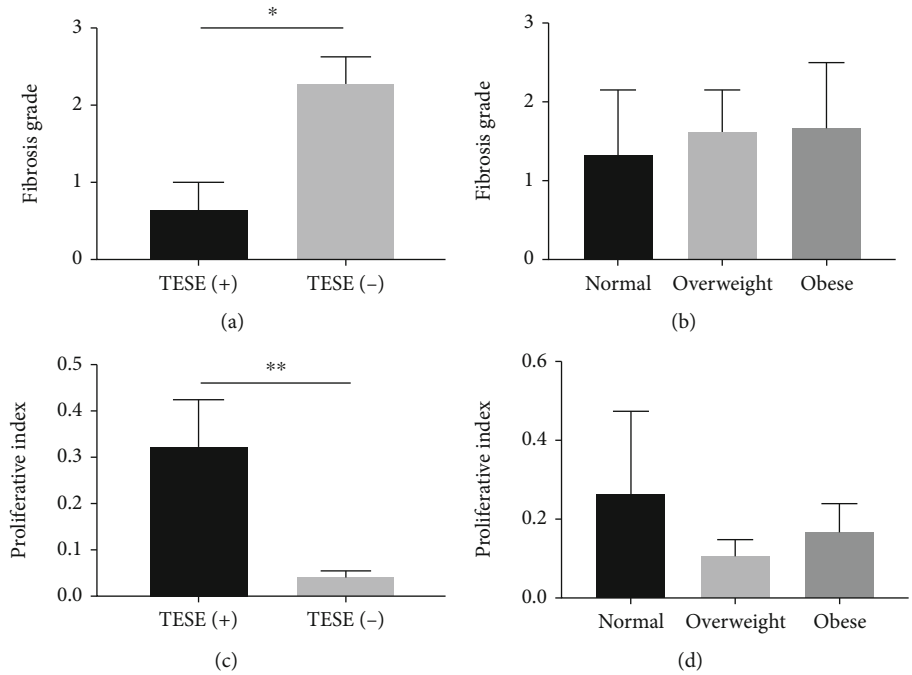

Figure 4: Fibrosis grade according to m-TESE positivity (a) and BMI (b). Proliferative index according to m-TESE positivity (c) and BMI (d).

Table 1: Relationship between the histologic type and PI.

Histologic type	PI (± SD)
Hypospermatogenesis	0.70 ± 0
Maturation arrest	0.23 ± 0.01
Germ cell aplasia	0.05 ± 0.06
Fibrosis	0 ± 0

apoptosis. Apoptotic TUNEL positive cells were observed in cases of maturation arrest and hypospermatogenesis. These cells were thought to belong to the spermatogenic series according to their location in the seminiferous tubule epithelium. In our study, significant results were obtained between the success of sperm retrieval with m-TESE and TUNEL positivity. It is noteworthy that sperms were obtained by m-TESE in all cases containing TUNEL positive cells. This suggests that TUNEL positivity may be a proof of the presence of spermatogenesis in the testis tissue. TUNEL positivity was detected in neither of the unsuccessful m-TESE cases, and the fact that most of these cases were patients with a diagnosis of germ cell aplasia makes this result consistent.

In addition to germ cell apoptosis in the testis, proliferation is also one of the mechanisms evaluated in many studies on infertility and related factors [33]. One of the markers used for this purpose is PCNA. PCNA expression in human testicular tissue has been shown to be in the nuclei of pale type A, type B spermatogonia, and primary spermatocytes [34]. In a study examining spermatogonial proliferation in azoospermia, the tubules in the mixed atrophic group were defined as normal, spermatocytic arrest, and spermatogonial arrest and were scored separately; decreased PCNA expression was observed in the same order [35]. In the study performed in idiopathic hypospermatogenesis cases, the balance between cell proliferation and cell death was examined by PCNA immunohistochemistry and in situ TUNEL methods. It was suggested that the underlying mechanism was increased apoptosis rather than a deterioration in proliferation [36]. In the present study, the PI results obtained by PCNA immunohistochemistry were the highest in hypospermatogenesis samples, and a significant decrease in maturation arrest, germ cell aplasia, and fibrotic tissue samples, respectively, was found in accordance with the literature. In addition, the fact that the PI was found to be significantly higher in the m-TESE positive group compared to the negative group indicates that the PI might be a numerical data that can support testicular histology in sperm retrieval from the testis in azoospermic patients.

Obesity is also one of the factors that increase testicular apoptosis. Studies on the effect of obesity on the testis tissue at the cellular and molecular levels have been demonstrated by animal studies [37–39]. Animals that were genetically obese or that were obese by being fed with a high-fat diet were used in these studies. In a study comparing both models, the apoptotic index was found to be higher in both groups compared to the controls. The degree of obesity was shown to be important in its effect on fertility, hormone and semen parameters, and spermatogenesis in both animal and human studies. Both genetic mutations and environmental factors are involved in the effect of obesity on fertility in humans. It has been concluded that it can be treated with lifestyle changes and that this may also have reversible effects on fertility [38].

In our study, obesity, that is, BMI, was shown to have no significant effect on TUNEL positivity and PI. In the literature, data on this subject are based on animal studies. To

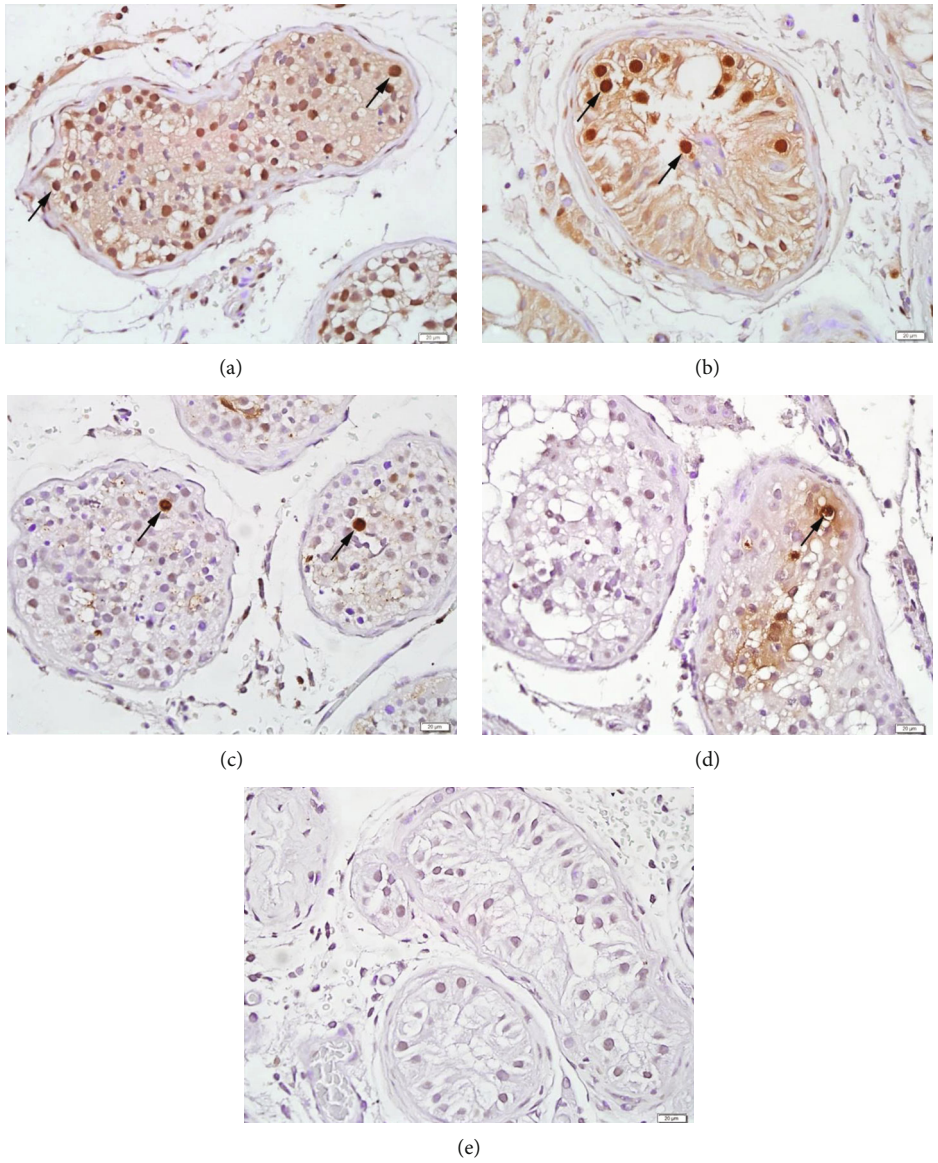

Figure 5: PCNA immunohistochemistry: (a) PCNA positive cells (arrows) in a case of hypospermatogenesis and (b) PCNA positive cells (arrows) in a case of maturation arrest. TUNEL immunohistochemistry: (c) TUNEL positive cells (arrows) in a case of hypospermatogenesis, (d) TUNEL positive cell (arrow) in a case of maturation arrest, and (e) negative control for TUNEL staining. Bars: 20 μm.

Table 2: Distribution of TUNEL positivity according to the histological type.

Histological type	Number of TUNEL positive cases	Number of TUNEL negative cases
Hypospermatogenesis	1	0
Maturation arrest	3	1
Germ cell aplasia	0	6
Fibrosis	0	1

Table 3: Distribution of TUNEL positivity according to m-TESE success.

	Number of m-TESE positive cases	Number of m-TESE negative cases
Number of TUNEL positive cases	4	0
Number of TUNEL negative cases	1	7

our knowledge, there is no human study in the literature examining the relationship between obesity and apoptosis and PI. Studies in humans mostly focus on the effects of obesity on reproductive hormones and semen parameters [40–43]. Most studies are examining the link between BMI and male infertility in terms of serum hormone levels [44].

There are many studies showing a negative relationship between BMI and total and free testosterone levels [45]. Decreased testosterone levels in humans have been associated with infertility, and obesity is one of the causes [46, 47]. Except for a few number of studies that showed decreased serum levels of gonadotropins, FSH, and LH released from the pituitary gland, most of the studies showed no significant change [48–50]. This was thought to be due to the opposing effects of rising estrogen levels and falling testosterone levels, resulting in no change in gonadotropin levels. In our study, it was concluded that BMI did not have a significant effect on serum FSH, LH, and testosterone values in NOA patients, in accordance with the literature.

The probability of factors being predictive in sperm retrieval by surgical methods in nonobstructive azoospermic patients, such as FSH, LH, testosterone, prolactin, age, BMI, testicular volume, and testicular morphology, has been evaluated in many studies [12, 14–16]. In a study in which TESE was applied, the sperm retrieval rate was 50%, and none of these factors was predictive [13]. In a study evaluating TESA results, it was concluded that BMI had no effect on sperm retrieval, and BMI was negatively correlated with testosterone and not correlated with FSH and LH [51]. In an m-TESE study, the sperm finding rate was 41.1%, and there was a significant correlation between the sperm finding rate and FSH, LH, and mean testicular volume, but no significance was found with BMI [52]. In a study examining the effect of obesity on the sperm availability rate in NOA patients who underwent TESA/ICSI, it was found that the testosterone levels of obese NOA patients were significantly lower, but there was no significant difference between FSH, LH levels, and sperm availability. In another study, it was concluded that there was no significant effect of FSH and BMI on TESE success in azoospermic men [53]. In a clinical retrospective study examining the data of NOA patients, no significant difference was found in the sperm retrieval rate by m-TESE between normal BMI, overweight, and obese groups [54]. In our study, FSH and LH levels were found to be higher than normal, and testosterone levels were found to be between normal ranges in both groups with successful and unsuccessful m-TESE, and no difference was found between the two groups in terms of reproductive hormones, age, and BMI.

As a result, we concluded that FSH, LH, testosterone, and BMI did not have a significant effect on the success of m-TESE in NOA patients. The fact that statistically significant results were not obtained may be due to the small size of the samples, so it would be appropriate to compare these values with a larger number of samples in our future study. The fact that the TUNEL and PCNA methods correlated with the success of m-TESE in a way that supports testicular histology suggests that these two methods are promising to give an idea about the probability of finding sperm with m-TESE, especially if the first procedure is unsuccessful, and a second procedure is planned. The effect of BMI on apoptosis and proliferation in testicular tissue has been demonstrated by animal studies. To our knowledge, our study is the first on this subject in human tissue. It has limitations as it is a preliminary study. Our study was prospective and interrupted by the pandemic period. The sample size was limited due to the postponement of m-TESE, which is an elective operation. However, we aim to conduct more extensive further studies on this subject in the future.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article. Raw data are available from the corresponding author upon reasonable request.

Ethical Approval

Approval was obtained from Marmara University Clinical Research Ethics Committee (09.2019.365).

Consent

Written consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

EK, SŞ, and AK are responsible for the design of the work and interpretation of the data. EK performed histological experiments, and AK performed m-TESE procedures. EK wrote the paper. SŞ was involved in drafting the work and critically revising the manuscript. EK, SŞ, and AK have approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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