ANDROLOGY

Original Article

Apoptosis in testicular tissue of rats after vasectomy: evaluation of eNOS, iNOS immunoreactivities and the effects of ozone therapy

Vazektomi sonrası rat testiküler dokusunda apoptois: eNOS, iNOS immünoreaktivitesi ve ozon terapinin etkilerinin incelenmesi

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ABSTRACT

Objective: We aimed to investigate the changes in endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression and apoptotic index in rat testicular tissue, as well as serum and seminal plasma sex hormone levels after vasectomy, and the effect of ozone therapy (OT).

Material and methods: Adult male Wistar rats were used (n=6 per group). Control (G1), sham for 4 weeks (G2) or 6 weeks (G3), orchiectomy at the 4th (G4) or 6th (G5) week after left vasectomy, orchiectomy at the 4th (G6) or 6th (G7) week after bilateral vasectomy, orchiectomy after 6 weeks OT following left (G8) or bilateral (G9) vasectomy, orchiectomy after 6 weeks OT (G10).

Results: In the left testes, while there were increases in eNOS and iNOS immunoreactivity and apoptotic indexes in G4 and G5, no changes were observed in contralateral testis. These values increased in G6 and G7, while OT inhibited these parameters in the left testis of G8 and both testes of G9. Sex hormone levels did not show any changes after vasectomy and ozone therapy.

Conclusion: While OT was found to be protective against some parameters mentioned above under stress conditions, it seemed to cause some harmful effects when used in healthy conditions.

Key words: Apoptosis; eNOS; iNOS; ozone therapy; vasectomy.

ÖZET

Amaç: Rat testiküler dokusunda vazektomi sonrası serum ve seminal plazma seks hormonları düzeyi yanı sıra endotelyal nitrik oksit sentaz (eNOS) ve indüklenebilir nitrik oksit sentaz (iNOS) ekspresyonu değişiklikleri ve apoptotik indeksi değişikleri ve ozon terapinin (OT) etkisini araştırmak.

Gereç ve yöntemler: Yetişkin erkek Wistar sıçanları (n=6 her grupta) kullanıldı. Kontrol (G1), 4 hafta (G2) ve 6 hafta (G3) sham operasyonu, sol vazektomi sonrası 4. hafta (G4) ve 6.haftada (G5) orşiektomi, bilateral vazektomi sonrası 4. hafta (G6) ve 6. haftada (G7) orkiektomi, plasebo ikili vazektomi, sol (G8) ve bilateral (G9) vazektomi sonrası 6 hafta ozon terapi sonrası orşiektomi 6 hafta sonra ve 6 hafta OT (G10) sonrası orşiektomi grupları oluşturuldu.

Bulgular: G4 ve G5'de sol testiste eNOS ve iNOS immünoreaktivitesinde ve apoptotik indekslerde artış varken karşı testiste hiçbir değişiklik gözlenmedi. OT G8'in sol testisinde ve 9'un her iki testisinde bu parametreleri inhibe ederken, G6 ve G7 artış izlendi. Seks hormon düzeyleri vazektomi ve ozon tedavisi sonrası herhangi bir değişiklik izlenmedi.

Sonuç: OT stres koşulları altında yukarıda bahsedilen bazı parametrelere karşı koruyucu olduğu tespit edilirken, sağlıklı koşullarda uygulandığında bazı zararlı etkilere neden olabileceği gözlendi.

Anahtar kelimeler: Apoptozis; eNOS; iNOS; ozon terapi; vazektomi.

Introduction

Vasectomy is a reliable and effective contraception method and its usage is increasing in men all over the world.[1] Rarely, some complications, such as bleeding or hematoma, or infections such as acute epididymitis, can be observed in the short term period.[2] Long-term complications are congestive epididymitis and scrotal pain, but these complications are rare.[3] Additionally, after surgery, there may be an increase in the antisperm antibody for a few years and an increase in immune complex that recovers three months after surgery.[4,5] Despite these possible complications, vasectomy is both a reliable and effective method when compared to other contraception methods. The

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most important subject that is emphasized by patients regarding this technique is whether it is reversible. Ross et al.[6] reported that approximately 1-3 out of every 1000 men who underwent vasectomy wanted to reverse the process in the following years. The recovery rate of these patients, which was evaluated by pregnancy rate, ranged from 30% to 60%.[7]

While the causes of infertility after vasovasostomy are thought to be vasectomy induced apoptotic and nitric oxide (NO) related changes in testicular tissue and their effects on spermatogenesis, the reason for this lower recovery rate is not clear. Apoptosis, described as programmed cell death, allows the continuance in the balance of construction-destruction throughout the germ cell life cycle. Likewise, NO has an important role in the control of testicular functions. While a low NO concentration plays a role in many physiological processes, a high NO concentration can lead to DNA damage and cell death.[8] Moreover, there are some publications reporting that a low concentration of NO improves sperm motility and is effective for the continuation of activity. Other publications report that a significant increase in NO levels may occur under pathologic conditions, such as testicular torsion.[9-11]

Recently, oozone therapy, which is performed with a mixture of gas no more than 5% O_3 (ozone) and no less than 95% O_2 (oxygen), is used in the treatment of many diseases. Ozone is an unstable gas, formed by the combination of three oxygen atoms in a cyclic structure. Although ozone is not a radical molecule, it is the third most potent oxidizer after fluorine and persulphate. Furthermore, when used in appropriate concentrations, some important effects, such as up-regulation of the antioxidant enzymes, immune modulation by activating the neutrophils and cytokine synthesis and increasing tissue oxygenation and neoangiogenesis occur.[12] Therefore, ozone therapy can be used in the treatment of some diseases, such as chronic cerebral and cardiac ischemia, rheumatoid arthritis, Crohn's disease, ostemyelitis, diabetic food lesions and peritonitis.

The aims of the present study were to evaluate inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expressions and apoptosis in the rat testes, changes in serum and seminal plasma gonadotropins and sex steroid levels after vasectomy and to observe the effects of ozone therapy on those changes by time.

Material and methods

This study was approved by Ethical Committee for Experimental Research on Animals (09/227162).

A total of 60 adult male Wistar rats (the mean body weight 352.5±37.67 g) were used in the study. They were kept at 25±2°C temperature and 32±7% humidity in a 12 h light/dark cycle. Rats were provided pelleted food and water ad libitum.

Rats were randomly divided into 10 groups, and each group was comprised of 6 rats. Study groups were described as following;

Group 1: Control group (G1).

Group 2: Sham group in which the left testicles of the members were removed and re-placed into the scrotum just after skin incision, and orchiectomy was performed at the end of the 4 weeks (G2).

Group 3: Sham group in which the left testicles of the members were removed and re-placed into the scrotum just after skin incision, and orchiectomy was performed at the end of the 6 weeks (G3).

Group 4: Bilateral orchiectomy was performed at the 4th week after left vasectomy (G4).

Group 5: Bilateral orchiectomy was performed at the 6th week after left vasectomy (G5).

Group 6: Bilateral orchiectomy was performed at the 4th week after bilateral vasectomy (G6).

Group 7: Bilateral orchiectomy was performed at the 6th week after bilateral vasectomy (G7).

Group 8: Bilateral orchiectomy was performed after 6 weeks ozone therapy following left vasectomy (G8).

Group 9: Bilateral orchiectomy was performed after 6 weeks ozone therapy following bilateral vasectomy (G9).

Group 10: Bilateral orchiectomy was performed after 6 weeks ozone therapy (G10).

All surgical procedures were performed under ketamine/xylazine (40/10 mg/kg, intramuscular) anesthesia. A lower abdominal midline incision was used for the vasectomy. After removing the tissues through the incision, the vas deferens was displayed. After removing a segmental bite, both sides were ligated with 5/0 silk. No complications, including bleeding and hematoma formation, were observed in the operation site.

Ozone therapy was performed with an ozone-oxygen gas mixture produced using an ozone generator (Evozone BasicPlus, Germany) on alternate days at 0.7 mg/kg intraperitoneally (i.p.). The dosage was set according to our previous experimental

studies. This application was performed with an inert syringe just after the ozone gas was generated.

Pathological analysis

The testicle tissues were fixated in 4% buffered paraformaldehyde for 48 hours. Later, they were processed considering the routine tissue processing procedure and embedded in paraffin wax. Tissue sections of 4-5 μm thickness were taken with a rotary microtome. Histopathological changes were evaluated under binocular light microscope, and photomicrographs were taken. In indirect immunoperoxidase tests, iNOS and eNOS immunoreactivities were investigated using commercial polyclonal rabbit anti iNOS and rabbit anti-eNOS primary antibodies, and all of the test steps were implemented according to the immunoperoxidase kit protocol (Labvision, Thermo Fisher Scientific, USA). Briefly, endogenous peroxidase activity was blocked by incubating tissue sections in 0.3% H_2O_2 for 10 minutes. Then, sections were incubated in normal goat serum for 7 minutes at room temperature, and they were incubated with a primary antibody (eNOS or iNOS) for one hour. After labeling with a biotinylated mouse anti-rabbit IgG secondary antibody, streptavidin peroxidase solution was applied. 3-Amino-9ethylcarbazole (AEC) chromogen and substrate solutions were applied, and immunoreactions were visualized under a binocular light microscope (Olympus BX51). The level of apoptosis was investigated using the Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick End-labeling (TUNEL) reaction considering the commercial kit protocol (in situ apoptosis detection kit, TaKaRa Bio. Inc, Shiga, Japan). Immunoreactivity and apoptotic cell count was performed at ×40 magnifications by using light microscopy.

Histomorphometric analysis

For histomorphometric analysis of the immunopositive cells, 10 photomicrographs representing the 1 mm² areas were taken from each testicle tissue using a calibrated Olympus BX51 microscope and DP25 digital camera. Afterwards, a 1 mm² grid scale containing 100 equal squares was prepared, and it was overlaid with the previously obtained photomicrographs of eNOS, iNOS immunoperoxidase staining and *TUNEL* reaction. To obtain the quantitative data for iNOS and eNOS positive cells, immunopositively stained germ and Sertoli and Leydig cells in 10 out of 100 randomly selected squares were counted, and their arithmetic mean value was calculated. The apoptotic index was calculated using the mean *TUNEL* reaction positive germ cells in the whole 1 mm² area for each testis tissue.

Biochemical analysis

Serum FSH, LH, total testosterone, estradiol and inhibin B levels and seminal plasma testosterone and estradiol levels were measured by the ELISA method and using USCN Life Science Inc (Wuhan, China) diagnostic kits.

Statistical analysis

For statistical calculations, the Statistical Package for Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA) was used. The arithmetic mean and standard deviation of the data were determined, and the Kruskal-Wallis test post hoc Dunn test was used to compare the groups. P values less than 0.05 were accepted as statistically significant.

Results

Changes of eNOS in Testes Tissue

eNOS immunoreactivity was observed mainly in early and late round spermatids in all experiment groups. Otherwise, spermatogoniums were weakly stained. Sertoli cells and Leydig cells also expressed eNOS, but the latter was lesser in number (Figure 1a-c).

It was found that eNOS immunoreactivity of the left testicle was similar among G1, G2, G3, G8, G9, and also among G4, G5, G6, G7, G10. However, eNOS immunoreactivity was found to be statistically higher in the rats that had only vasectomy performed (G4, G5, G6, G7) and applied only ozone therapy (G10) ($p<0.0001$) (Table 1). At the same time, we thought that levels of eNOS were not time-dependent because there was no difference between 4 and 6 weeks.

Right testicular eNOS immunoreactivity was found to be similar in G1, G2, G3, G4, G5 and G9 ($p=0.278$). These findings showed that left vasectomy had no effect on eNOS immunoreactivity in the contralateral site. Additionally, it was demonstrated that ozone therapy had a protective effect on both testicles when bilateral vasectomy was performed (G9). Moreover, the higher values in G6 and G7 compared to all other groups, except G10, revealed that vasectomy led to an increase in eNOS immunoreactivity in the testicles.

eNOS values were statistically higher on the left side where vasectomy was performed than on the intact right testes in G5 ($p=0.028$). Therefore, we thought that vasectomy led to an increase in eNOS immunoreactivity on the operation side, while this effect did not reflect to the contralateral testicular tissue**.** In the group that ozone therapy was used alone (G10), the eNOS level increased more than in all of the other groups. Although ozone therapy had a protective effect on the left testicle after vasectomy, it caused an increase in eNOS values on the intact right testicle (G8).

Changes of iNOS in testicular tissue

iNOS immunoreactivities were detected in the early and late spermatids, Sertoli and interstitial Leydig cells, respectively (Figure 1d-f).

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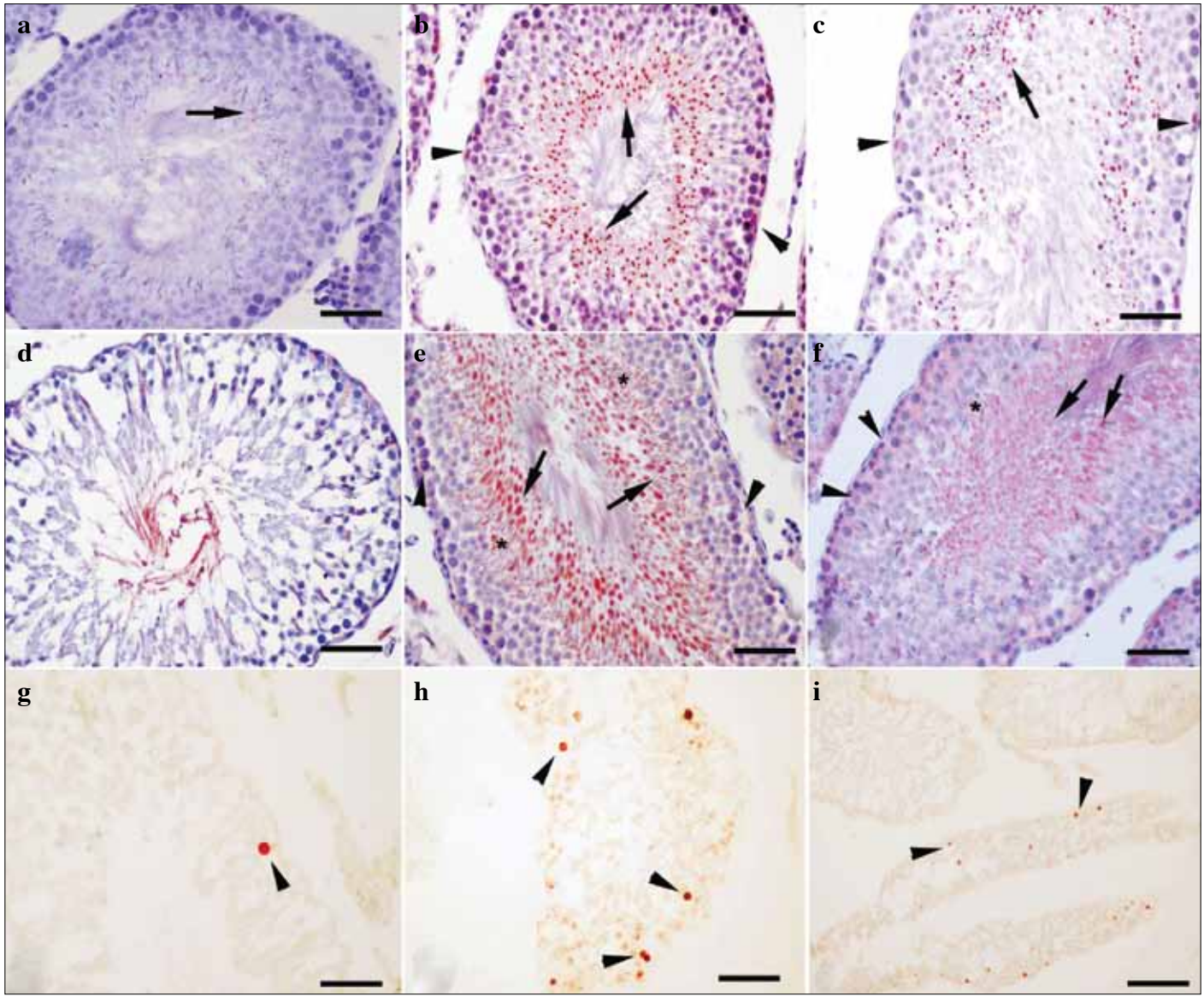

Figure 1. eNOS and iNOS immunoreactivity and apoptotic changes in testes. eNOS antigen immunopositive reactions in the early and late round spermatids (arrows) and Sertoli cells (arrowheads). ABC indirect immunoperoxidase test, rabbit anti-eNOS antibody, Mayers hematoxylin counterstaining, Bar=180 µm. (a) Control Group 1; (b) Bilateral vasectomy 6 weeks, Group 7; (c) Bilateral vasectomy + ozone therapy Group 9)]. iNOS expressions in the spermatids, spermatogonia (arrows) and in Sertoli cells (arrowheads). Rabbit anti-iNOS antibody, ABC indirect immunoperoxidase test, Mayers hematoxylin counterstaining, Bar=180 µm; (d) Control Group 1; (e) Bilateral vasectomy 6 weeks Group7; (f) Ozone therapy Group 10. Apoptotic cells in seminiferous tubule epithelia. TUNEL reaction, no counter staining, Bar=180 µm; (g) Control Group1; (h) Bilateral vasectomy 6 weeks Group7; i: Ozone therapy Group 10

Only vasectomy (G4, G5, G6, G7) or only ozone therapy application (G10) led to a significant increase in iNOS immunoreactivity on the left testicular tissue when compared to the control and sham groups (Table 1). iNOS immunoreactivity in the right testicle was higher in G6 and G7 (to which only vasectomy was applied), and in G10 (to which only ozone therapy was applied) than in all of the other groups ($p<0.0001$). Additionally, while the increase in iNOS was suppressed, evidently with ozone therapy in the right testicular tissues (to which vasectomy was applied) (G9), this therapy caused a clear iNOS increase in G8 in which vasectomy was not performed.

iNOS values in the left testicle, where vasectomy was applied, were found to be statistically higher than in the intact right testicle in G4 ($p=0.046$). These findings suggested that while vasectomy led to an increase in iNOS immunoreactivity on the

Table 1. eNOS and iNOS immunoreactivity values on the left and right testicular tissue in the study groups (Mean±SD and min-max), *eNOS G5 right vs. left testis values (p=0.028), **iNOS G4 right and left testis values (p=0.046)

	eNOS		iNOS	
Group	**Right testis**	**Left testis**	**Right testis**	**Left testis**
G1	5±2.7 (2-8)	4.5±2.9 (1-9)	5±3.8 (1-12)	3.2±1.3 (1-5)
G2	7.5±2.2 (4-10)	6.8±2.9 (4-12)	6.8±4.5 (1-13)	6.8±3.6 (2-12)
G3	7.2±1.8 (5-10)	11.8±2.3 (8-14)	6.5±1.4 (4-8)	7.7±2 (4-9)
G4	11.3±2.4 (8-15)	17.5±6.2 (10-25)	7.7±3.1 (4-12)	13.5±5.2 (5-19)**
G5	8.5±2.8 (6-12)	16.8±7.4 (9-30)*	10.8±1.9 (9-14)	13.5±3.7 (7-18)
G6	17±3.1 (14-21)	21.8±5.2 (14-30)	21.3±2.9 (18-26)	18.5±5 (11-24)
G7	25.2±3 (21-30)	23.2±2.8 (21-28)	17.5±2.7 (13-21)	16.8±2.3 (13-19)
G8	16.2±0.8 (15-17)	9.8±6 (3-19)	12.7±3.5 (7-16)	6.3±1.2 (5-8)
G9	9.2±2.6 (6-13)	7.8±3.8 (3-12)	5.7±1.6 (3-8)	5.3±2.7 (1-8)
G10	30.7±5.1 (26-38)	29±5.2 (24-36)	20.1±7.1 (12-28)	19.7±3.4 (17-26)

eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase

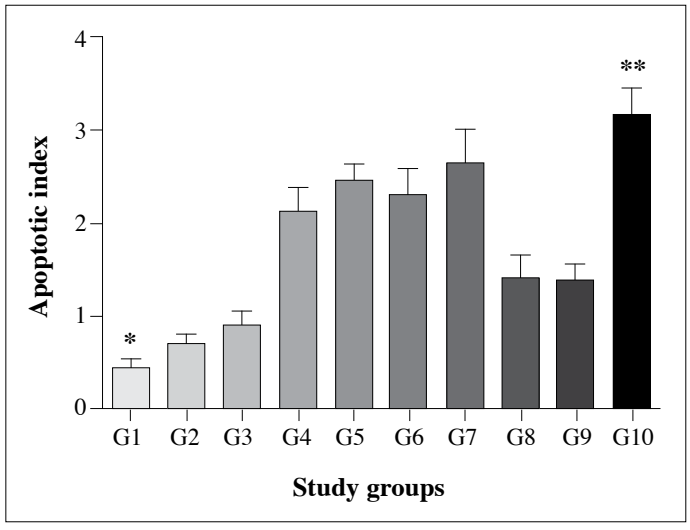

Figure 2. Left testis apoptotic index values in study groups (Means)* control vs. G5, G6, G7, G10, **G10 vs. G1, G2, G3

operation side, this effect did not reflect to the contralateral testicular tissue.

Apoptotic Changes in Testes Tissue

Apoptotic changes were mainly observed in the epithelial cells and Sertoli cells in the seminiferous tubules (Figure 1g-i).

The evaluation of apoptotic changes was conducted on the left testicle. Apoptotic index values were found to be close to each other in the control (G1), sham groups (G2 and G3) and ozone therapy groups with vasectomy (G8 and G9). Similarly, apoptotic index values were not statistically different among the vasectomy only groups (G4, G5, G6, G7) and the ozone therapy only group (G10); however, the apoptotic values of these groups showed a statistically meaningful difference from G1 ($p<0.001$). Particularly, ozone therapy used on a healthy rat caused a more characteristic increase on the apoptotic index than all other groups, even higher than that observed in the rats who received vasectomy (Figure 2). Additionally, in this study, it was observed that the elapsed time after vasectomy had no effect on apoptotic index.

Table 2. Seminal plasma testosterone and estradiol levels in study groups (Mean±SD and min-max)

Group	Testosterone (ng/mL)	Estradiol (pg/mL)
G1	131.9±59.8 (69.7-206.9)	2.4±1.2 (0.93-4.05)
G2	74.7±58.6 (20.0-173.3)	1.6±1.8 (0.04-4.20)
G3	47.3±28.3 (4.8-87.6)	2.62±2.6 (0.61-7.65)
G4	63.8±30.6 (11.9-91.3)	11.2±15.4 (0.38-36.9)
G5	100.1±74.3 (12.2-195.3)	2.7±2.8 (0.04-6.32)
G6	163.1±67.8 (106.6-289.9)	4±3.3 (0.38-8.65)
G7	102.1±32.9 (67.7-164.6)	4.2±3.2 (0.0-9.73)
G8	160.3±51.1 (91.3-217)	8.3±13.5 (0.3-35.62)
G9	189.8±105.8 (47.4-274.9)	5.8±3.5 (1.72-9.18)
G10	128.4±37.2 (93.7-195.3)	6.2±9.2 (0.89-24.6)

Biochemical evaluation

There were no significant differences in seminal plasma, testosterone, and estradiol levels, and we did not observe differences in serum FSH, LH, testosterone and estradiol levels among study groups. However, the serum inhibin B level was statistically significantly lower in G6, G7 and G8 than in other groups (Table 2 and Table 3).

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Table 3. Serum hormone levels (Mean±SD and min-max), *p=0.019 vs. other groups

Group	FSH (IU/mL)	LH (IU/mL)	Testosterone (ng/mL)	Estradiol (pg/mL)	Inhibin B (pg/mL)
G1	5±3.32 (2.3-11.4)	0.94±0.37 (0.5-1.5)	1191.2±677.8 (175.3-1984)	31.1±11.6 (13.3-40.2)	133.1±66.5 (60.8-207.2)
G2	4±1.57 (2.3-5.8)	2.07±1.15 (0.4-3.4)	755±538.6 (307.2-1760)	60.2±66 (22.1-193.7)	96.1±55.5 (37.5-193.7)
G3	3±0.98 (1.5-4.6)	1.38±0.88 (0.3-2.5)	947.7±469.2 (427.2-1745)	36±13.1 (25.8-61.4)	132±75.3 (44.1-264.9)
G4	4.54±1.8 (2.1-7.6)	5.11±5.95 (1.4-16.9)	365.5±339.2 (50.4-1005)	33.5±6 (27-42.2)	98.1±32.8 (67.4-148.8)
G5	4.06±1.7 (1.7-5.9)	1.74±0.67 (0.68-2.3)	1047±1033.3 (344.5-3017)	41.1±16.8 (28.1-72.8)	98.3±49.1 (59.2-189.5)
G6	3.96±1.76 (1.9-6.6)	2.49±2.48 (0.8-6.8)	1192.7±1181 (407.9-3565)	40.7±6.8 (31.3-50.8)	67.8±29.9 (34.6-114)*
G7	2.79±2.27 (0.2-5.5)	1.27±0.68 (0.3-2.3)	984.1±696 (331.7-1929)	39.2±8.5 (27.7-52.5)	51.1±14.5 (31.6-67.4)*
G8	1.71±1.44 (0.1-3.3)	1.57±0.64 (0.9-2.4)	699±475.6 (217.2-1282)	39±12.7 (30.6-64.1)	51.3±16.6 (33.6-70.3)*
G9	5.96±2.83 (3.3-9.4)	0.95±1.06 (0.2-3.1)	1059.8±584.6 (521.1-2119)	29.7±4.1 (22.9-34.9)	106.7±60.8 (36.5-197.3)
G10	3.88±2.16 (2.0-7.3)	1.22±0.65 (0.6-2.3)	1319.1±1095 (311.2-2922)	38.5±4.5 (30.8-44.7)	105.7±40.3 (40.8-148.4)

FSH: Follicle stimulating hormone; LH: Luteinizing hormone

Discussion

Vasectomy is a safe and effective male contraceptive method. However, the individuals who choose to have this procedure may return to the physician with requests for fertility in the future. Although microsurgical vasovasostomy should be an appropriate surgical approach in patients who wish to have children after vasectomy, Bekler et al.[13] reported that the continuity of the vas with this technique was approximately 79%, and the pregnancy rate was 44% in the 9-14 year post-vasectomy period. The reasons for this lower success rate have not been identified clearly yet. In a human study, seminiferous epithelium degenerations due to increased hydrostatic pressure in the seminiferous tubules after vasectomy were identified.[14] Additionally, McVicar et al.[15] reported that the spermatid/Sertoli cell ratio per unit area was found to be decreased without reducing the number of Sertoli cells in individuals who underwent vasectomy. The effects of vasectomy showed differences in various species in experimental studies. While increased intraluminal pressure has affected spermatogenesis in dogs, leukocyte infiltration caused by an autoimmune orchitis in guinea pigs was caused by this situation.[16] Contrarily, the degeneration of the seminiferous epithelium due to the immune complex deposition along the basal membrane in rabbits, and the testicular atrophy related to the spermatic granuloma after vasectomy in hamsters was found to affect spermatogenesis.[17,18] Lue et al.[19] found that the apoptosis ratio was increased in the spermatide of hamsters after vasectomy.

Kubota et al.[20] reported that vasectomy results in damage to spermatogenesis in adult rats, which may induce germ cell apoptosis. e-NOS and i-NOS may play a critical role in the germ cell apoptosis after vasectomy. Basaran et al.[21] showed an increase in eNOS and iNOS immunoreactivity after testicular torsion in rats.

There is a close relationship between the NO level and sperm fertilizing capacity.[22] While NO is in normal physiologic limits, it demonstrates positive effects on capacitation and acrosome reactions of spermatozoa, and it can cause lipid peroxidation and spermatozoa membrane protein damage with reactive oxygen species in higher concentrations.[23] At the same time, reduced ATP synthesis and inhibition of spermatozoa DNA biosynthesis and mitochondrial respiration were effective in the spermatozoa damage.[24] Oktem et al.[25] reported that the apoptotic pathway was a complex process for protecting the testicular integrity, as well as the effectiveness of eNOS and iNOS. An increase in the number of apoptotic germ cells was associated with increased NO levels and decreased total antioxidant capacity.[26]

After ozone reacts with polyunsaturated fatty acid (PUFA), hydrogen peroxide having a much shorter half-life, lipid oxidation products having long half-lives, such as lipohydroxides (LOO•), alcoxyl radicals, isoprotanes and alkenes are formed. Despite the fact that the Reactive Oxygen Substances (ROS) are always accepted as being harmful, this perspective has changed dramatically because it plays a role as a signal transduction regulator and an important mediator in host defense and immune response in physiologic amounts.[12] However, excessive amounts of ROS can cause an increase in the levels of some toxic substances such as peroxynitrite ($O=NOO^-$) and hypochlorite anion (ClO^-). To take advantage of the biological effects, the applied ozone dose should be set very carefully.

In our study, the increased apoptosis after vasectomy was found to be statistically significant in rat testicular tissue. We observed that ozone therapy alone led to more increased apoptosis than vasectomy. However, ozone therapy after vasectomy prevented apoptosis. While eNOS and iNOS immunoreactiviy

in testicular tissue increased after vasectomy, this augmentation was blocked by ozone therapy. However, ozone therapy alone led to an increase in the number of eNOS and iNOS positive cells than vasectomy alone. On the contrary, vasectomy did not affect eNOS and iNOS immunoreactivies on the contralateral testicular tissue.

Alexander and Tung[27] observed that vasectomy had no effect on reproductive hormones. Miller et al.[28] also reported no differences in androgen levels taken from the seminal vesicle after vasectomy. Moreover, Smith et al.[29] reported that there was no correlation between micro epidydimal sperm aspiration (MESA) success rates and serum FSH, LH, testosterone and inhibin B levels in patients with primary obstructive azoospermia. Sinha Hikim[30] claimed that gonadotropin releasing hormone analog deprivation caused an increase in apoptosis. In the present study, while we found increased apoptosis after vasectomy, no differences were found in the levels of gonadotropins and sex steroids, nor in the serum or in seminal plasma. However, only the serum inhibin B level was found to be statistically significantly lower in the bilateral vasectomy group and unilateral vasectomy applied ozone therapy group than in all other groups. We thought that decreased inhibin B levels in the bilateral vasectomy group may be due to increased apoptotic activity in Sertoli cells. However, because it was not observed after unilateral vasectomy, and despite the fact that the ozone therapy led to a decrease in apoptosis, no changes were observed in inhibin B levels, we concluded that vasectomy did not affect inhibin B levels. Therefore, we believe that the increased apoptotic changes after vasectomy were not due to the changes in gonadotropin levels.

We thought that ozone therapy plays a role depending on the pathologic status of tissues. Briefly, ozone therapy was found to be protective against some parameters mentioned above under stress conditions, while it obviously seemed to cause some harmful effects when applied in healthy conditions. For instance, as found in the present study, if the oxidant status is prevalent, ozone therapy might prevent injury. However, if there is no challenge to the oxidant/antioxidant balance, then ozone might be deleterious itself. Because we did not focus on the oxidant/antioxidant balance indicators in this work, it is hard to make a definitive conclusion. However, it may be speculated that both ozone therapy and vasectomy-induced factor(s) may increase the expression of eNOS and iNOS and the apoptotic process when applied alone, although these effects most likely occur through different transcriptional mechanisms. They neutralize each other's effect when applied together and are likely to be mediated by the molecular interaction between the related molecules. NO seems to play a pivotal role in this interaction causing the paradoxical effect mentioned above.

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