Original Article

The role of nitric oxide on spermatogenesis in infertile men with azoospermia

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ABSTRACT

Objectives: The underlying pathophysiological mechanisms of azoospermia is still unclear. The aim of the study was to evaluate nitric oxide synthase (NOS) isoforms and free radical release in testicular sperm extraction (TESE) in infertile men with azoospermia.

Materials and methods: The study included 40 men (mean age: 37.2±2 years; range 25 to 55 years) with azoospermia which were divided into two groups: spermatozoa-present (n=20) and spermatozoa-absent (n=20). Testicular samples were examined morphologically, immunohistochemically, and biochemically. The TESE samples were examined according to number of mast cells stained with toluidine blue; immunohistochemically with three types of NOS isoforms, and free radicals were measured with chemiluminescence method, respectively.

Results: Endothelial NOS (eNOS) reaction in spermatozoa-present group was considerably higher than spermatozoa-absent group (p<0.001). Compared to the spermatozoa-present group, inducible NOS (iNOS) reaction was higher than the spermatozoa-absent group (p<0.05). Neuronal NOS (nNOS) reaction was only prominent in Leydig cells in both groups. Mast cells increased (p<0.05) in the interstitial area surrounding seminiferous tubules in spermatozoa-absent samples. Superoxide radical generation in spermatozoa-present samples was significantly lower (p=0.0003). The peroxynitrite ratio in spermatozoa-absent samples was significantly higher (p=0.0038).

Conclusion: These results suggest that eNOS, iNOS, and mast cells play an important role in spermatogenesis process in azoospermic men.

Keywords: Male infertility, mast cell, nitric oxide, oxidative stress, testicular sperm extraction.

Fertile men are defined as males whose partner conceived within 12 months after stopping use of contraception.[1] Male fertility requires the production of a sufficient number of mature and motile spermatozoa. A defect in any of these parameters may cause male infertility. Azoospermia is the absence of spermatozoa in the ejaculate. Surgical methods have been developed to retrieve spermatozoa from the epididymides and the testes of patients.[2] After sperm acquisition, intracytoplasmic sperm injection (ICSI) can be applied which results in higher fertilization rates.[3] Sperm retrieval (SR) is based on the type of azoospermia which is either obstructive (OA) or non-obstructive (NOA). Testicular sperm extraction (TESE) is the technique of choice for NOA.[2]

Several studies have shown that high levels of reactive oxygen species (ROS) are related with male infertility.[4-6] Excessive amounts of ROS produced by leukocytes and immature spermatozoa can cause damage to the normal spermatozoa.[6-8] Situations in which ROS are

associated also involve mast cell activation.[9] Nitric oxide (NO) is one of the ROS which regulates the physiology of the reproductive function. It is synthesized by nitric oxide synthase (NOS) which exists in three known forms: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS).[10]

In the present study, we aimed to evaluate NOS isoforms and free radical release in TESE in infertile men with azoospermia.

MATERIALS AND METHODS

TESE and sperm analysis

In this prospective cohort study, ejaculate samples were taken from male patients who applied for *in vitro* fertilization (IVF) and were evaluated according to the 2010 World Health Organization (WHO) sperm analysis criteria[1] between Februrary 2011 and June 2013. The samples were taken twice from each patient with sexual abstinence between two and seven days. The specimens were examined and analyzed with a Makler® sperm counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel). When there was no sperm in the pellet of the centrifuged sample, male patients were referred for TESE operation. Testicular biopsy was taken and a specimen size of 2 to 3 mm was obtained, where the seminiferous tubules were opaque and dilated which were later examined morphologically. The study protocol was approved by the Istanbul Bilim University, Medical Faculty, Ethics Committee (2011/SAG-022). The study was conducted in accordance with the Declaration of Helsinki.

Histological and immunohistochemical analysis

A total of 40 male patients (mean age: 37.2±2 years; range 25 to 55 years) were included for persistent azoospermia. The study did not take into account the patient's medical history, physical examination, or hormonal profile. Testicular biopsy samples in infertile men with azoospermia were divided into two groups as follows: spermatozoa-present [sperm (+) group] (n=20) and spermatozoa-absent [sperm (-) group] (n=20).

Testicular samples were fixed in the Bouin's solution. The samples were embedded in paraffin. The sections were, then, stained with the Masson's trichrome to examine connective tissue and stained with Toluidine blue to define mast cells, and NOS isoforms and were examined immunohistochemically, while the seminiferous tubules were graded according to the Johnson score.[11]

Masson's trichrome staining protocol for connective tissue

The slides were deparaffinized, immersed in graded alcohol and finally rinsed in water. The slides were, then, fixed with the Bouin's fixative. Then, each slide was immersed and left in Weigert's iron hematoxylin solution for approximately 10 min. Each slide was passed through acid fuchsin, acetic acid, phosphomolybdic acid, and Aniline blue solution, respectively. Finally, the slides were rinsed in 2% acetic acid and washed with distilled water. Once the slides were dehydration with Clear® xylene, they were ready to be examined under microscopy.[12]

Toluidine blue staining protocol for mast cells

The sections were deparaffinized and hydrated with distilled water. The slides were, then, immersed in toluidine blue. After staining, the sections were passed through distilled water, alcohol, and xylene respectively over a period of time. For mast cell counting, five fields were selected from each slide at ×200 magnifications and the obtained values were evaluated statistically.[11]

Immunohistochemical analysis

The sections (3 μm) were deparaffinized with xylene and rehydrated with ethanol and finally with water. Antigen retrieval was accomplished in the Decloaking Chamber™ in a citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked with 3% H_2O_2. Blocking reagent was applied to each slide, followed by 5-min incubation at room temperature in a humid chamber. Sections were incubated for overnight at 4°C with rabbit polyclonal iNOS antibody, eNOS antibody, and nNOS. The sections were biotinylated goat anti-rabbit antibodies. Slides were washed in PBS after which streptavidin peroxidase label reagent was applied. The colored product was incubated in 3, 3'-diaminobenzidine,

tetrahydrochloride, dihydrate (DAB). The slides were counterstained with Mayer's hematoxylin. The intensity and the distribution of NOS staining were scored by histological (H-score) value.

Determination of free radicals

For the determination of the superoxide anion, NO, and peroxynitrite radicals in testicular samples of azoospermia, we used the chemiluminescence (CL) method. The CL measurements were made by using a luminometer. The TESE sample was divided into two and put into vials containing 2 mL of PBS+HEPES buffer (0.5 M PBS containing 0.02 M 4-(2-hydroxyethyl), piperazine-1-ethanesulfonic acid; pH 7.4). Superoxide radicals were quantitated after the addition of lucigenin enhancer for a final concentration of 0.2 mM. The CL measurement of NO is based on the reaction of hydrogen peroxide and NO to peroxynitrite. However, peroxynitrite can also form in TESE sample by a reaction between superoxide and NO.[13] To differentiate

between peroxynitrite and NO levels, the measurement was done from the same test tube after addition of 0.5 mM carboxy-PTIO-potassium salt (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt), a NO scavenger. The difference between the measurements indicated the level of peroxynitrite forming in testicular samples. All counts were obtained at 1-min intervals for a total period of 5 min. The results were given as the area under the curve (AUC) of relative light unite and corrected for wet tissue weight (AUC of rlu/mg tissue).

Statistical analysis

Statistical analysis was performed using the GraphPad Software Inc., CA, USA. Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency. The Student's t-test was used to compare two independent groups. The Mann-Whitney U test was used to analyze significant differences between the groups. A p value of <0.05 was considered statistically significant.
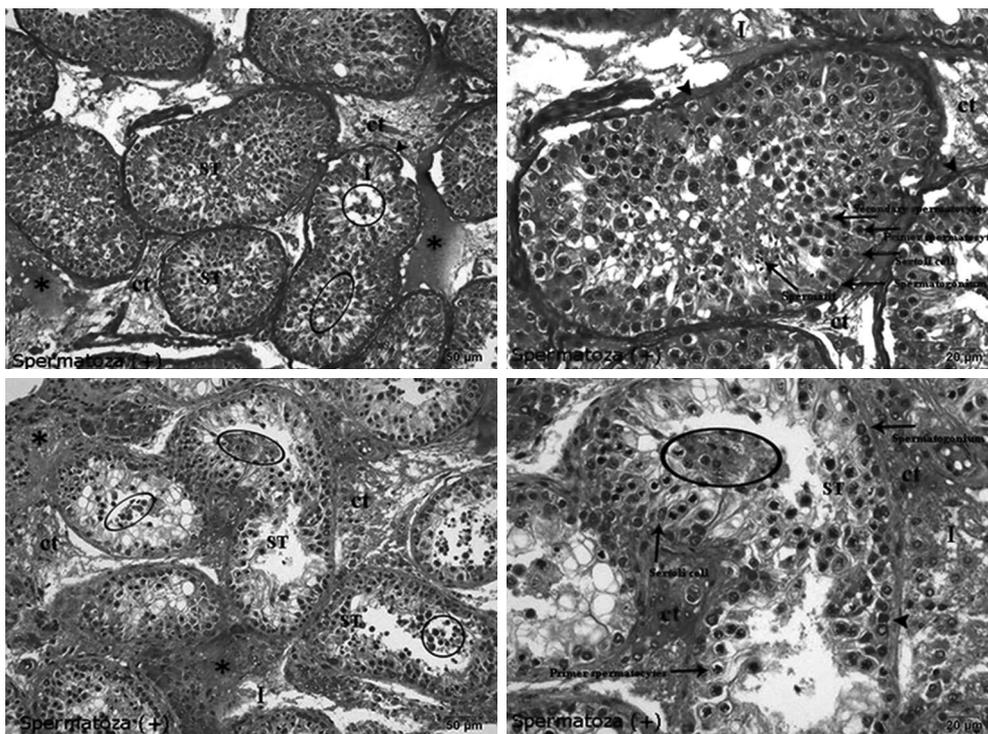

Figure 1. Spermatogonium (→), primary spermatocytes (→), secondary spermatocytes (→), Sertoli cells (→), spermatid (→), basal membrane (➤), connective tissue (ct), interstitial area (I), structures (0) in lumen of seminiferous tubules (ST) and hemorrhage area (*) in testis tissue of spermatozoa (+) groups. Masson's trichrome. Scale bar: 20 μm, 50 μm.

RESULTS

Histopathology of testicular samples

An abnormal morphological structure was determined in the testicular tissues in both groups of azoospermic men. The spermatozoa-present group revealed that the epithelium of seminiferous tubules was both lined with Sertoli and spermatogenic series; spermatogonium, primary and secondary spermatocytes, spermatids were present. The tubules were surrounded by a fine basal membrane of collagen fibers and, within the interstitial area, there was hemorrhage containing a number of Leydig cells (Figure 1).

In spermatozoa-absent group, the structure of seminiferous tubules was seen to have degenerated and hyalinized. An extensive intratubular and peritubular hyalinization with an absence of germ cells was observed. Spermatogonia were not detected, thus, proving that spermatogenesis process did not occur. Spermatogonia were absent; however, Sertoli cells were present (Sertoli cell-only syndrome as the spermatozoa-absent group). The tubular basal membranes were thickened and wrinkled, the lumens became shrunk, and the spermatogonia did not develop along with loosening in the tubular membrane and edema (Figure 2).

All cases were ranked according to the modified Johnson's scoring system (Tables 1, 2). Histological examination of the testicular structure of the spermatozoa-absent group showed that 20% did not include seminiferous epithelium and, of the remaining 80%, 30% included only Sertoli cells, 30% included spermatogonia cells, and 20% included many spermatocyte cells. In the spermatozoa-present group, 5% included only spermatogonia cells, 15% included few spermatocyte cells, and 80% of the spermatid cells progressed to the spermatozoa stage completing the spermatogenic series.

Index of mast cells

The mean number of mast cells in the interstitial area surrounding the seminiferous tubules (Figure 3) significantly increased in the
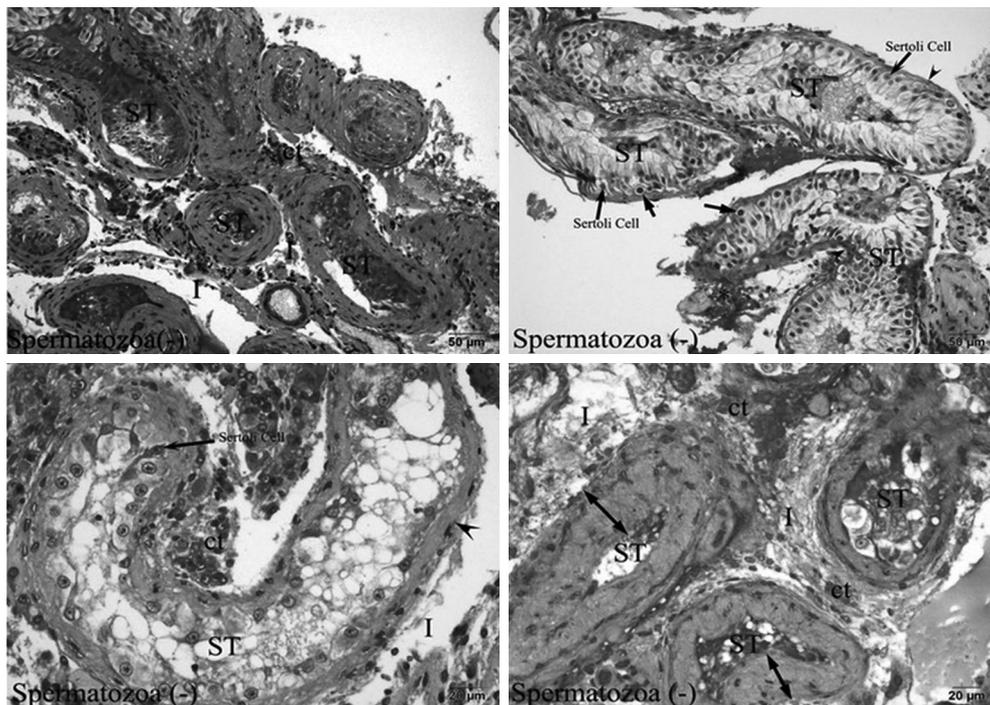

Figure 2. Deteriorated, degenerated and hyalinized seminiferous tubules (ST), spermatogonium (➜), Sertoli cells (→), thickened basal membrane (↕ , ➤), the tubule cells seperated from basal membrane (→), connective tissue (ct), interstitial area (I) and hemorrhage area (*) in testis tissue of spermatozoa (-) groups. Masson's trichrome. Scale bar: 20 μm, 50 μm.

Table 1. Ranking of spermatozoa (+) group according to the modified Johensen scoring system

	Score histological criteria (According to the modified Johensen scoring)									Total
	No seminiferous epithelium	No germinal cells, Sertoli cells only	Spermatogonia only	No spermatozoa or spermatids, few spermatocytes	No spermatozoa or spermatids, many spermatocytes	No spermatozoa, no late spermatids, few early spermatids	No spermatozoa, no late spermatids, many early spermatids	Less than ten spermatozoa per tubule, few late spermatids	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium	
	1	2	3	4	5	6	7	8	9	
Sperm (+)	0	0	1	3	1	1	6	5	3	20
Sperm (-)	4	6	6	0	4	0	0	0	0	20
Total	4	6	7	3	5	1	6	5	3	40

	n	%
Johensen 9	3	15
Johensen 8	5	25
Johensen 7	6	30
Johensen 6	1	5
Johensen 5	1	5
Johensen 4	3	15
Johensen 3	1	5
Johensen 2	0	0
Johensen 1	0	0
Total	20	100.0

Table 2. Ranking of spermatozoa (-) group according to the modified Johensen scoring system

	Score histological criteria (According to the modified Johensen scoring)									
	No seminiferous epithelium	No germinal cells, Sertoli cells only	Spermatogonia only	No spermatozoa or spermatids, few spermatocytes	No spermatozoa or spermatids, many spermatocytes	No spermatozoa, no late spermatids, few early spermatids	No spermatozoa, no late spermatids, many early spermatids	Less than ten spermatozoa per tubule, few late spermatids	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium	Total
	1	2	3	4	5	6	7	8	9	
Sperm (+)	0	0	1	3	1	1	6	5	3	20
Sperm (-)	4	6	6	0	4	0	0	0	0	20
Total	4	6	7	3	5	1	6	5	3	40

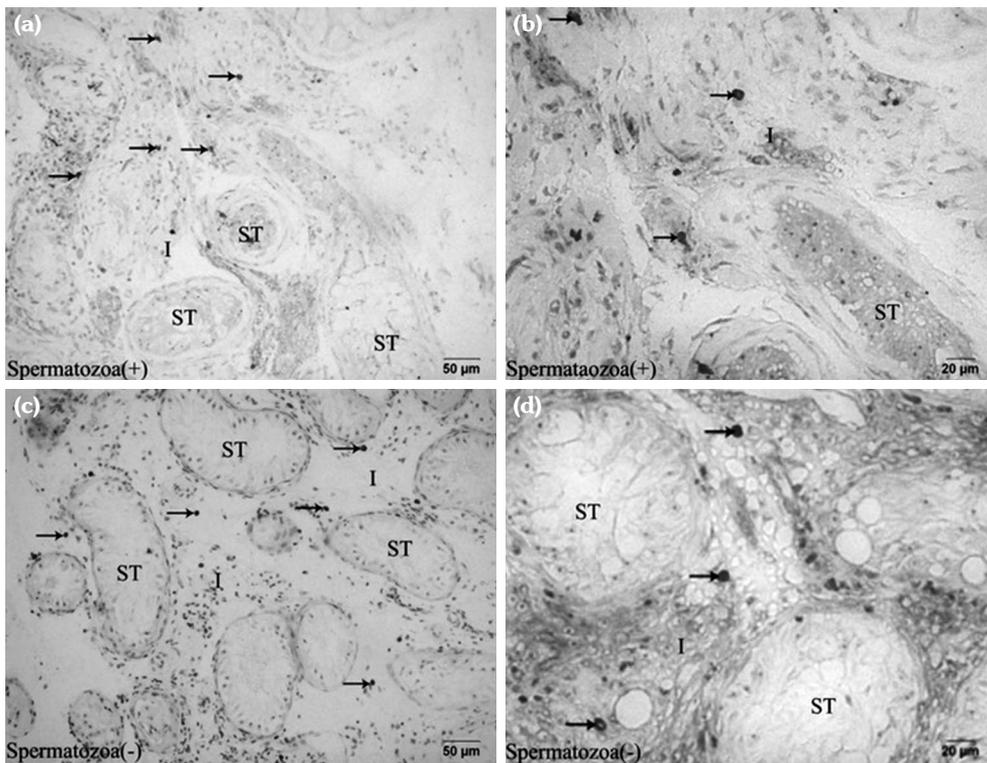|	n	%
Johensen 9	0	0
Johensen 8	0	0
Johensen 7	0	0
Johensen 6	0	0
Johensen 5	4	20
Johensen 4	0	0
Johensen 3	6	30
Johensen 2	6	30
Johensen 1	4	20
Total	20	100.0

Figure 3. Mast cells (→) in the interstitial area (I) surrounding seminiferous tubules (ST) in **(a, b)** spermatozoa (+) and **(c, d)** spermatozoa (-) group. Toluidine blue. Bar: 50 μm, Scale Bar: 20 μm.

spermatozoa-absent group (18.5±7.3) compared to the spermatozoa-present group (9.7±5.6, p<0.05, Figure 4).

NOS immunohistochemistry

In the spermatozoa-present group, the mean eNOS reaction was considerably higher (127.9±4.9) than the spermatozoa-absent group (105±4.6,

Figure 4. The index of mast cells at the testis tissue of spermatozoa (+) and spermatozoa (-) groups.

* p<0.05 vs spermatozoa (-) group.

p<0.001, Figure 5). eNOS reactions occurred in the myoid cells around the seminiferous tubules, and also Sertoli cells and primary spermatocytes in the seminiferous tubules. In addition, eNOS reaction was also prominent in the spermatogenic cell line of the seminiferous tubules. This reaction could also be seen in the Leydig cell and blood vessels in the interstitial area (Figures 6, 7).
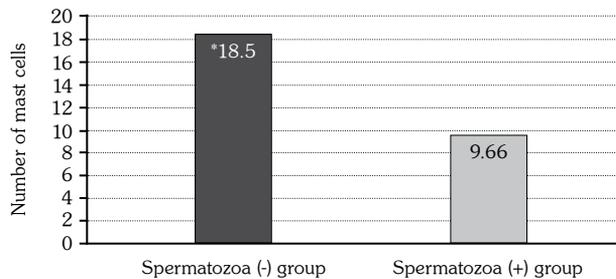
In the spermatozoa-present group, the mean iNOS reaction (228.7±6.7) was lower compared to the spermatozoa-absent group (254.4±8.5, p<0.05, Figure 5). The iNOS reaction was higher, compared to the eNOS reaction. The iNOS reaction was prominent in the seminiferous tubules consisting of spermatogonial cells and Sertoli cells. In addition, the iNOS reaction was observed in myoid cells and Leydig cells (Figures 6, 7).

There was no significant difference in the mean concentration of nNOS reaction between the both groups (92.5±3.8 *vs.* 87.5±5.3 in the spermatozoa-present and spermatozoa-absent
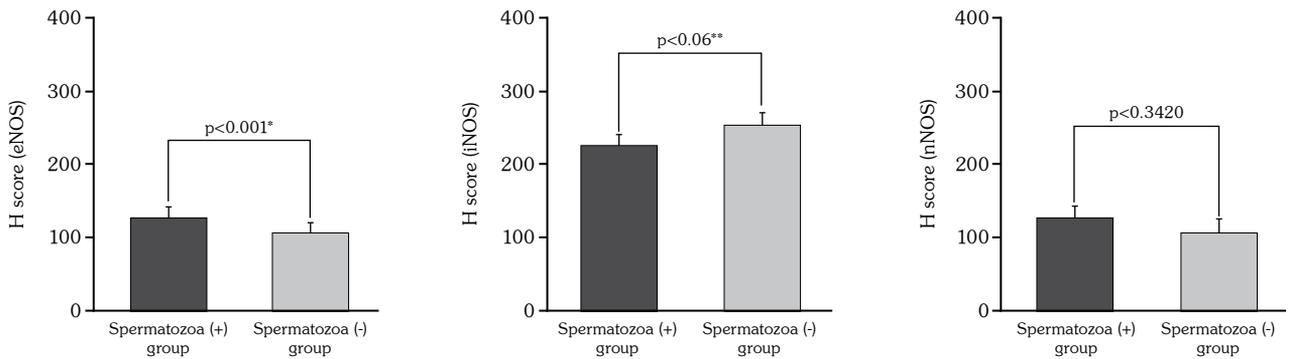
Figure 5. Distribution of the eNOS, iNOS and nNOS reactions at the testis tissue of spermatozoa (+) and spermatoza (-) groups.

eNOS: Endothelial nitric oxide synthase: iNOS: Inducible nitric oxide synthase; nNOS: Neuronal nitric oxide synthase; * p<0.001 vs spermatozoa (-) group; ** p<0.05, vs spermatozoa (+) group.

groups, respectively; Figure 5). The nNOS reaction was only prominent in Leydig cells in the interstitial area; however, there was no reaction observed in spermatogonia and Sertoli cells (Figures 6, 7).

Free radical measurement results

Superoxide radical generation in the spermatozoa-present group was significantly lower than spermatozoa-absent group (10.3±2.3 rlu/mg
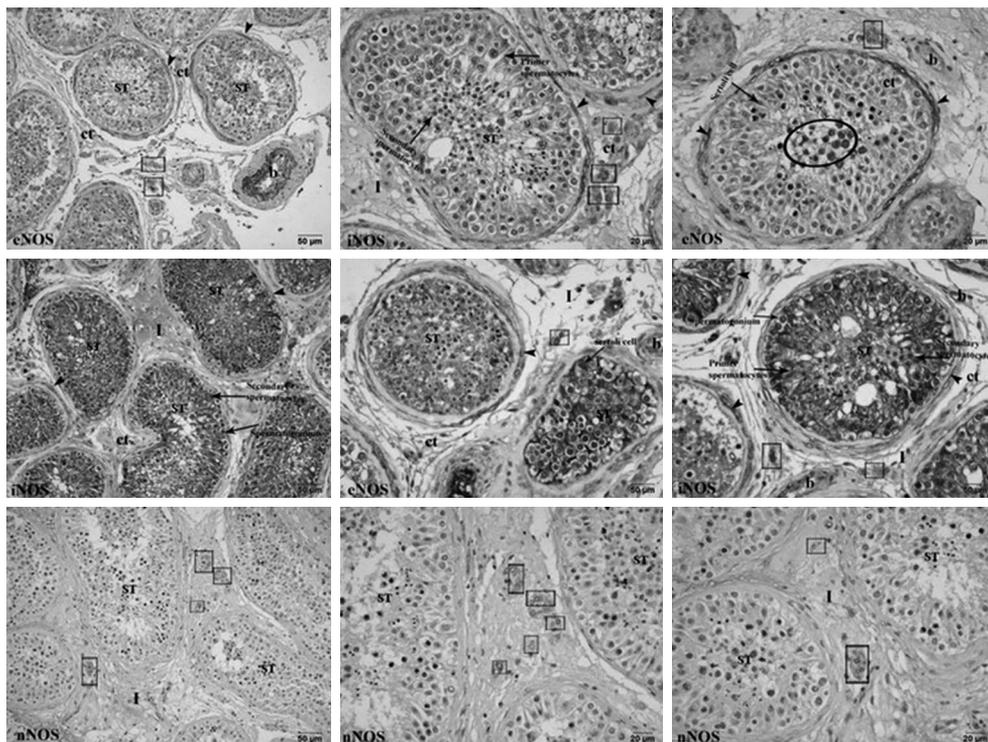

Figure 6. Spermatogonium (→), primary spermatocytes (→), secondary spermatocytes (→), Sertoli cells (→), spermatid (→), basal membrane (➤), connective tissue (ct), blood vessel (b), Leydig cell (☐), interstitial area (I) and structures (0) in lumen of seminiferous tubules (ST) of spermatozoa (+) groups. eNOS, iNOS and nNOS reactions. Scale bar: 20 μm, 50 μm.

eNOS: Endothelial nitric oxide synthase: iNOS: Inducible nitric oxide synthase; nNOS: Neuronal nitric oxide synthase.
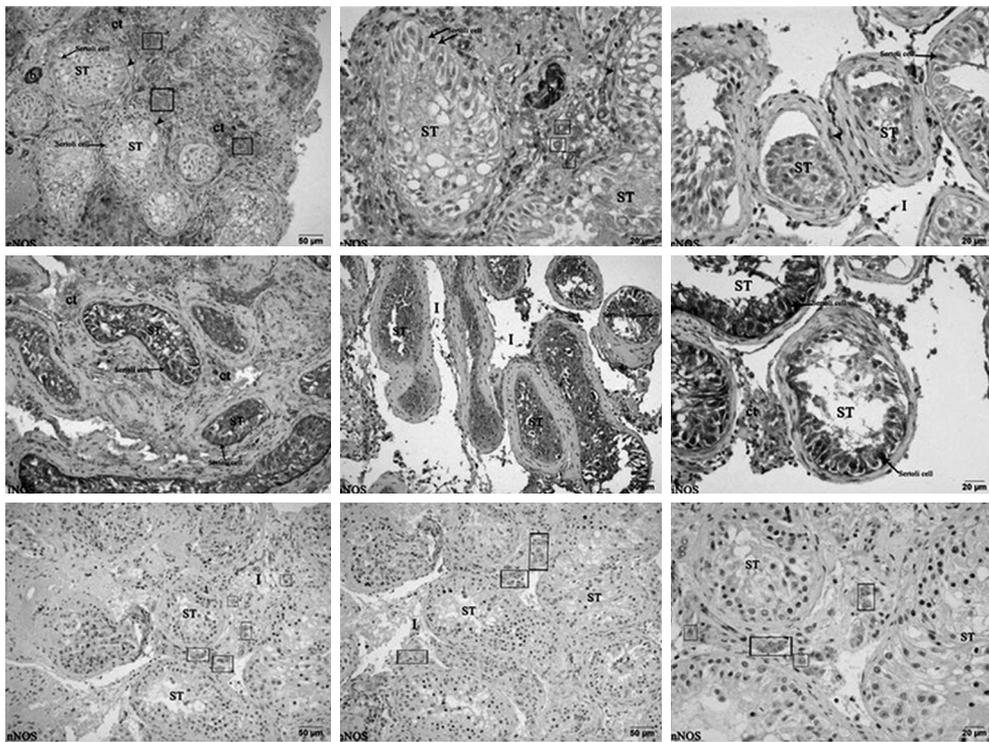

Figure 7. Sertoli cells (→), basal membrane (➤), connective tissue (ct), blood vessel (b), Leydig cell (□) and interstitial area (I) in testis tissue of spermatozoa (-) groups. eNOS, iNOS and nNOS reactions. Scale bar: 20 µm, 50 µm.

eNOS: Endothelial nitric oxide synthase: iNOS: Inducible nitric oxide synthase; nNOS: Neuronal nitric oxide synthase.

tissue *vs.* 19.4±7.2 rlu/mg tissue, respectively; p=0.0003, Figure 8). The mean NO release of spermatozoa-present group was 111.9±65.5 rlu/mg tissue. In the spermatozoa-absent group, it was 29.8±18.5 rlu/mg tissue (p<0.0001) (Figure 9). The mean peroxynitrite ratio in the spermatozoa-absent group (42.5±17.8%) was significantly higher than the spermatozoa-present group (19.1±11.5%; p=0.0038) (Figure 10).
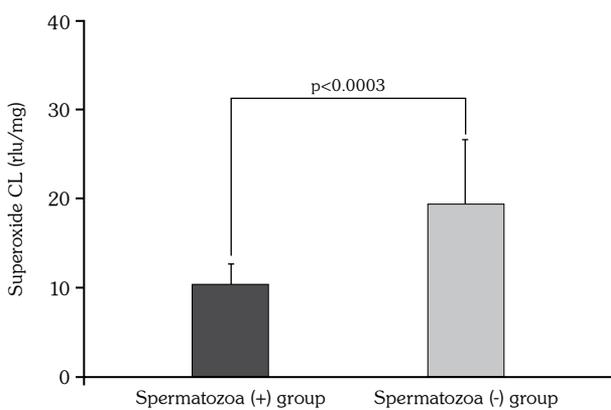

Figure 8. Superoxide radical generation testicular tissue of spermatozoa (+) and spermatozoa (-) group.
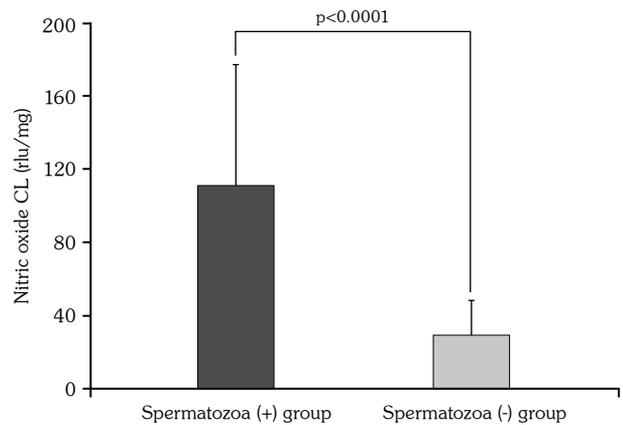
CL: Chemiluminescence; p=0.0003 vs spermatozoa (-) group.

Figure 9. Nitric oxide chemiluminescence in testicular tissue of spermatozoa (+) and spermatozoa (-) group.
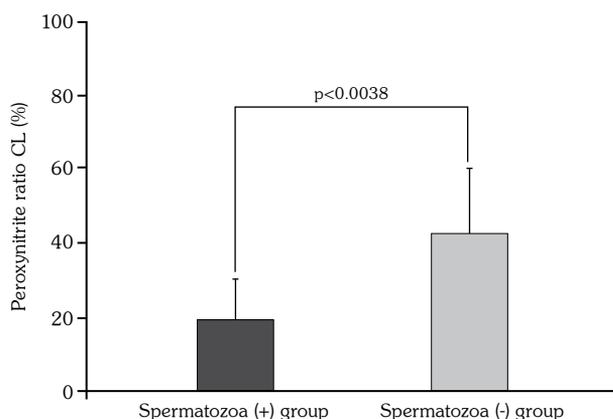
CL: Chemiluminescence; p<0.0001 vs spermatozoa (-) group.

Figure 10. Peroxynitrite ratio in testicular tissue of spermatozoa (+) and spermatozoa (-) group.

CL: Chemiluminescence; p=0.0038 vs spermatozoa (-) group.

DISCUSSION

The main and novel findings of this study showed the isoforms of NOS enzymes and markers of oxidative stress in infertile men with azoospermia. In the spermatozoa-absent group, oxidative stress increased along with an increase in mast cells, leading to inflammation. The iNOS-mediated oxidative stress combined with inflammation promoted spermatogenesis in the spermatozoa-absent group. Both patient groups, with and without spermatozoa, released NO produced by different NOS isoforms, making it important pathophysiologically. In 5 to 10% of infertile men with NOA, azoospermia occurs.[2]

Research on testicular torsion in rat testicular tissue demonstrated that detorsion caused testicular tissue damage, such as histopathological degenerative changes, lack of maturation in the germinal cells, interstitial edema, disorganization in the seminiferous tubule, and the increase in apoptotic index in rat testis tissue.[14] Another study showed that testicular torsion-detorsion led to enhancing apoptosis in germ cells and increased the Bax messenger ribonucleic acid expression (proapoptotic).[15] In other studies, it was demonstrated that a duration of 4 h, 720° rotation of the spermatic cord, and detorsion caused a significant overproduction in the levels of NO content, lipid peroxidation, and myeloperoxidase activity (an indicator of neutrophil accumulation) in the rat testicles.[16-18] This resulted in permanent loss of spermatogenesis.[16,19] In our study, histopathological examination revealed

that, in the azoospermic men (spermatozoa-absent group), the seminiferous tubule epithelium thickened and contained only Sertoli cells with decreased Johnson's scores.

Spermatogenesis and steroidogenesis are controlled by a master switch (gonadotropin-releasing hormone pulse generator) in which two separate feedback systems enable independent control of androgen (luteinizing hormone [LH]-testosterone) and sperm production (follicle-stimulating hormone [FSH] inhibin).[20] These hormones are known to influence the spermatogonia fate. Many studies can be found regarding lipopolysaccharide (LPS)-induced oxidative stress damage in testis, explaining how LPS-induced oxidative stress in mice testes and damaged germ cells, Leydig cells, and reduced testosterone levels.[10,21] Their removal induces germ cell apoptosis. In vitro studies in human testicular tissue materials have demonstrated the role of FSH and testosterone in the prevention of germ cell apoptosis suggesting that both hormones act as germ cell survival factors. The role of Sertoli cells in the regulation of the apoptotic mechanisms of germ cells is confirmed by the fact that the expression of Fas ligand in the testis is mainly localized in Sertoli cells.[22] In our study, in the spermatozoa-present group, primary spermatocyte and a few spermatid could be seen easily between the Sertoli cells. On the other hand, in the spermatozoa-absent group, tubules became degenerated, hyalinized, and germ cells were not detected. Germ cells that were produced including spermatogonia, spermatocytes, and spermatid somehow underwent apoptosis.

Oxidative stress activates iNOS in testis, resulting in excess NO generation. The NO is a powerful oxidant and plays a major role in testicular damage which is associated with overproduction of NO.[23] The NO is synthesized by NOS. There are three types of NOS, namely eNOS, iNOS, and nNOS, all of which are found in the testes. Germ cells express eNOS, whereas Leydig cells produce iNOS abundantly.[24] On the other hand, minor amounts of nNOS are produced by Sertoli cells. Despite the presence of these classical NOSs, a testis-specific NOS variant has been identified and entitled as the truncated form of nNOS (TnNOS) recently. Its expression is limited to the Leydig cells, strongly implicating its role in steroidogenesis.[25] In our study, there was

no significant difference in the concentrations of nNOS reactions in either group. The nNOS was also localized, particularly in the Leydig cells. In our opinion, the nNOS is related to and plays an important role in the production of the testosterone hormone in Leydig cells.

In their study, Ying et al.[25] reported that eNOS gene played a role in normal spermatogenesis and the genetic variants of eNOS gene might be potential genetic risk factors of spermatogenesis impairment in testis. These findings suggest that the variants of eNOS gene may modify the susceptibility to male infertility with impaired spermatogenesis. Our results showed that eNOS was necessary for spermatogenesis, iNOS could arrest spermatogenesis, and nNOS could act as a signal molecule for spermatogenesis. At this point, the following question arises: how does iNOS regulate spermatogenesis? Our results indicated that mast cells increased in the spermatozoa-absent group. Sezer et al.[26] demonstrated that increase of mast cell index was observed in the groups of infertile testis and high expression of iNOS in Leydig cells was associated with the highest mast cell index in Sertoli cell only syndrome, the lesion with the severest damage of the germ cell. It is known that mast cells activate fibroblast and promote collagen synthesis by producing and releasing proteolytic enzymes (i.e., trypsin) and other substances associated with inflammation and fibrosis.[27] In our study, particularly in the spermatozoa-absent group, increased collagen synthesis and narrowing in the tubular structure were observed. The increased numbers of mast cells in infertile males are seen both in the interstitium and around seminiferous tubules.[28] Increases in peritubular mast cells are associated with peritubular fibrosis.[27]

In our study, we stained three NOS isoforms immunohistologically, but measured only NO with the CL method. The NO can be also measured via nitro tyrosine or nitrite/nitrate concentrations, and other methods. In our histopathological examination, nNOS remained unchanged, but an increase in the eNOS was seen in the spermatozoa-present group with an increase in the iNOS in the spermatozoa absent-group. Biochemical measurements indicated that NO formation in the spermatozoa-present group was higher than the spermatozoa-absent group. These findings indicate that the source of the increased

NO formation via CL measurement is eNOS, while iNOS helps to mediate this increase.

In biological systems, superoxide and NO can react at a high rate and produce peroxynitrite radical, which is toxic to cell membranes and other cellular components.[29] In a study, high peroxynitrite levels were associated with decreased concentrations of Na^+/K^+-ATPase activity, Ca^{+2}-ATPase activity, and intracellular Ca^{+2} concentrations in the asthenozoospermic patients' spermatozoa compared to normozoospermic patients.[30] These results indicate that sperm membrane is destroyed via peroxynitrite formation and decreased motility and loss of sperm function in idiopathic asthenozoospermia. As shown in our study, the peroxynitrite ratio in the spermatozoa-absent group had more than doubled compared to the spermatozoa-present group, demonstrating that superoxide radical generation was significantly higher in the spermatozoa-absent group. Superoxide dismutase (SOD) activity, which is an enzyme, scavenges superoxide and converts it into hydrogen peroxide. In higher levels of pachytene spermatocytes, round spermatids and spermatozoa reflect a greater increase in the SOD than in the Sertoli cells. Some authors have shown that different categories of testicular cells display variable susceptibility to oxidative stress, and also superoxide radical generation.[31,32] Superoxide anion overproduction may be an important step in the cascade of ROS-related damage to spermatozoa, resulting in impaired semen parameters in patients with varicocele.[33] The increase in the ROS suggests that the condition of oxidative stress may cause an arrest in the spermatogenesis stage. Therefore, if we can eliminate oxidative stress, we can support sperm production and the arrest situation of the spermatogenesis can be removed.

The main limitations of this study are that we had no sufficient information about the testosterone levels, FSH, LH, and androgen hormone profile of the patients in the high oxidative stress group, which precluded commenting on the pituitary functions. In addition, based on these results, we cannot comment on the rate of fertilization, rate of implantation, and rate of ongoing pregnancy in the sperm-present group after IVF treatment.

In conclusion, our study results show that the increase of ROS plays an important role in

sperm production, and avoiding the conditions of exposure to oxidative stress and providing supportive therapies can lead to further stages of spermatogenesis from the stage of arrest. Avoiding oxidative stress and supportive therapies for spermatogenesis arrest may help to advance to the next stage. In patients with negative specimens according to the TESE, this precursor sperm cell (found or elongated smermatids) using round sperm injection (ROSI) or elongated sperm injection (ELSI) techniques provides an opportunity for the patient to have a child.

Declaration of conflicting interests

Funding

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