Journal of **R**eproduction & **I**nfertility
Volume 25, Issue no. 3
https://doi.org/10.18502/jri.v25i3.17012

Original Article

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Association of NOX5 Expression with Sperm Activity and Motility in Pathospermic Infertile Men

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Abstract

Background: The newest NOX isoform, NOX5, has been found in mammalian spermatozoa. Many physiological and pathological situations in spermatozoa are mediated by reactive oxygen species (ROS). NOX5 is the main source of ROS in spermatozoa. Our purpose was to investigate the changes in NOX5 expression and the effect of NOX5 expression on sperm motility, chromatin integrity, and oxidative status in oligoasthenozoospermic compared to normozoospermic men.

Methods: Semen samples were collected from 30 normozoospermic (NS) and 30 oligoasthenozoospermic (OAS) men. NOX5 protein expression in sperm samples was evaluated by immunohistochemistry and western blot. Oxidative stress status was evaluated by total antioxidant capacity (TAC), total oxidant capacity (TOC), and oxidative stress index (OSI) parameters. Chromatin integrity in spermatozoa was evaluated by toluidine blue staining.

Results: NOX5 expression levels were significantly higher in OAS group than in NS group ($p<0.001$). In addition, chromatin integrity was significantly higher in the OAS group in comparison to NS group ($p<0.001$). TAC levels were higher in the NS group, but OSI and TOC levels were significantly higher in OAS group ($p<0.001$). It was found that NOX5 protein expression was positively correlated with oxidative stress and chromatin integrity and negatively correlated with motility ($p<0.01$).

Conclusion: These results suggest that overexpression of NOX5 may be the source of excessive ROS production and oxidative stress injuries in oligoasthenozoospermic men. Considering that NOX5 expression is positively correlated with oxidative stress and chromatin integrity but negatively correlated with motility, it can be considered a biomarker to be used in assisted reproductive procedures.

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Received: 11, Feb. 2024
Accepted: 29, Jun. 2024

Keywords: Male infertility, NADPH oxidase 5, Reactive oxygen species, Sperm motility.
To cite this article: Daylan B, Korkmaz O, Tok OE, Cakici C, Yigitbasi T, Karahasanoglu A, et al. Association Of NOX5 Expression with Sperm Activity and Motility in Pathospermic Infertile Men. J Reprod Infertil. 2024;25(3):184-192.https://doi.org/10.18502/jri.v25i3.17012.

Introduction

The World Health Organization (WHO) defines infertility as the inability to conceive despite unprotected sexual intercourse for 12 months (1). Common causes of male infertility include various factors such as chronic stress, obesity, urogenital anomalies, metabolic diseases, reactive oxygen species, and genetic disorders (2, 3).

Reactive oxygen species, such as hydrogen peroxide, superoxide anion, and hydroxyl radical, are

small molecules produced from oxygen and can damage the sperm plasma membrane. Production of ROS by sperm cells has been associated with lipid peroxidation, DNA oxidation, poor sperm function, and low fertility (4, 5). Therefore, excessive production of ROS or decrease in sperm antioxidant defense system may cause infertility. Many studies show that redox activity is physiologically important in supporting normal sperm function (6, 7). ROS are important signaling molecules required for processes such as sperm capacitation, hyperactivation, acrosome reaction, sperm-oocyte fusion, and fertilization under physiological conditions, but their excess can impair sperm function (8-10). It has also been shown that oxidative stress caused by excessive ROS production is the main cause of male infertility and is closely related to apoptosis by disrupting DNA integrity (11, 12).

In humans, the NOX5 gene has been identified on chromosome 15. Although the presence of this gene and its enzyme has been identified in humans (13), dogs (14), and equines (15), surprisingly, it has not been identified in murine genomes (16). In addition to testis and sperm, NOX5 is expressed in many human fetal tissues (uterus, spleen, lymph nodes), pachytene spermatocytes, and endothelial cells (13, 15-17). Overexpression of NOX5 has been associated with various pathologies (18, 19). NOX5-derived ROS has been reported to cause a number of diseases such as cancer, cardiovascular and kidney diseases, as well as other pathological conditions such as pulmonary arterial hypertension, neurodegenerative diseases, and liver fibrosis (20, 21). In pathological conditions, ROS initiates oxidative stress damages such as DNA fragmentation, lipid peroxidation, decreased sperm function, and impaired fertility (22, 23). Despite the obtained evidence for the physiological and pathological effects of ROS in sperm, the enzyme(s) producing ROS still remain unclear. Therefore, the aim of this study was to investigate the effects of NOX5 expression levels, which have been proposed as a candidate for ROS production if sperm, on sperm motility and chromatin integrity in semen samples from normozoospermic (NS) and oligoasthenozoospermic (OAS) patients.

Methods

Population and semen collection: The current study was approved by the Ethics Committee of Istanbul Medipol University (10840098-604.01.01-E.16100). The study consisted of two groups including oligoasthenozoospermic men (n:30) and normospermic men (n:30). All participants, aged 20-45, were informed about the study and provided written informed consent. Semen samples were collected from the infertility treatment center at Medipol Mega University Hospital in Turkey. According to the standards established by the World Health Organization (WHO) in 2021, oligoasthenozoospermia is characterized by a sperm concentration below $16\times10^6/ml$, as well as progressive motility and total motility below 30% and 42%, respectively. Normospermic men are individuals with sperm concentrations of $\geq16\times10^6/ml$, normal sperm morphology ≥4%, and total sperm motility ≥42%. Sperm parameters were evaluated according to WHO guidelines (24). Based on the inclusion criteria, normozoospermic and oligoasthenozo-ospermic men who did not have any known medical conditions or were not taking any medication were included. Exclusion criteria were varicocele, urogenital tract infection, testicular tumor, history of radiotherapy or chemotherapy, leukocytospermia, and congenital or endocrine disorders. Patients with these conditions were not included in the study.

Semen analysis and preparation: Semen samples were collected in sterile containers after 3-5 days of sexual abstinence and analysied in accordance with WHO criteria (24). Afterwards, samples were incubated in an incubator at 37°*C* for 15 *min* to allow for liquefaction. Semen samples were centrifuged at 5000 *rpm* for 5 *min*, following the liquefaction process. Then, half of the pellet was stored at -80°*C* for western blot analysis and the supernatants were stored at -20°*C* for determination of total oxidant and total antioxidant levels. The other half of the pellet was washed with culture medium (HTF/HEPES, AR-1023) and centrifuged again at 5000 *rpm* for 5 *min*. Smear preparations were made, and sperm morphology in the normozoospermic and oligoasthenozo-ospermic groups was evaluated using Spermac staining and Diff-quick staining methods.

Measurement of TAC and TOC: Total antioxidant capacity was measured colorimetrically in seminal plasma samples. ABTS (2,2'-azino-bis (3-ethylbenzothi-azoline-6-sulphonic acid)) reagent was radicalized with hydrogen peroxide in the presence of buffer solution, keeping the pH constant. The resulting solution has a distinctive dark green to dark blue color. When seminal plasma was

added, the antioxidants present in the seminal plasma neutralized the ABTS radicals. As the antioxidants neutralized the ABTS radicals, the color of the solution lightened. Therefore, the color intensity of the solution is proportional to the total amount of antioxidants in seminal plasma. The absorbance of the solution was measured at 658 *nm*. The total antioxidant capacity of the sample was calculated using the absorbance and molarity data for the reference solution (25).

Total oxidant capacity was measured colorimetrically in seminal plasma. Fe_2SO_4 was dissolved in water and Fe^{+2} was released. Oxidants present in seminal plasma caused Fe^{+2} to be oxidized to Fe^{+3}. The xylenol orange reagent formed a complex with Fe^{+3}. The intensity of the color is proportional to the total amount of oxidants. Absorbance was measured at 658 *nm*. Using the absorbance values of the standard solution, the total oxidant capacity of the sample was calculated (26). OSI was calculated using the formula OSI= (TOS/TAS) x 10.

Evaluation of sperm chromatin integrity: The smears of washed sperm were fixed in a fixation solution (96% ethanol-acetone) at +4°*C* for 1 *hr*. After fixation, it was hydrolyzed in 0.1N HCl and then washed with dH_2O. It was then stained with 0.05% toluidine blue for 10 *min* and washed with dH_2O. The results were evaluated with a 100X objective lens microscope (Nikon Eclipse Ni; Nikon Japan). Sperm heads with intact chromatin integrity were observed as light blue, and those with disrupted integrity were observed as dark purple. At least 200 cells were counted in different areas of the slide preparation, and the results were reported as a percentage (27).

Immunohistochemistry staining: The smear preparations were fixed by keeping them in 4% paraformaldehyde (158127-5G; Merck, Germany) for 20 *min* at room temperature and then washed with phosphate buffered saline (PBS) (P0261; Merck, Germany). To block endogenous peroxidase activity in sperm, the preparations were kept in 3% hydrogen peroxide (H_2O_2) (CAS 7722-84-1; Merck, Germany) for 20 *min* at room temperature, followed by washing with PBS. Antigen retrieval was performed at 200 *watts* for 10 *min*. The preparations were washed with PBS after cooling. Then, the blocking step was performed. After the blocking step, the NOX5 primary antibody (cat no: ab191010, diluted 1/50; Abcam, UK) was added to the samples and incubated overnight at +4°*C*. After incubation, the samples were washed with PBS and incubated for 10 *min* at room temperature with a biotinylated solution. Next, the samples were washed with PBS and incubated for 10 *min* at room temperature with HRP-conjugated secondary antibody solution. After that, the samples were incubated in a 1:50 dilution of the DAB chromogen/substrate solution (ab64238; Abcam, UK) for 5 *min*. The samples were then counterstained by adding Mayer's hematoxylin (H9627; Merck, Germany) for 3 *min*. Then, the slide samples were washed with tap water and mounted using a mounting medium. As the final step, the regions with NOX5 protein expression in sperm were examined and scored under a light microscope (Nikon Eclipse Ni; Nikon Japan), and images were captured using a 100X objective lens (28).

Protein extraction and western blot analysis: Semen samples were stored at -80°*C*. For analysis, equal volumes of samples from the same group were pooled together. Semen samples from each group were centrifuged at 10,000×*g* for 15 *min* at 4°*C*. The supernatant was discarded and the cellular fraction was collected and homogenized using RIPA lysis buffer. The homogenized sample was centrifuged at 10,000×*g* for 15 *min* at 4°*C*, and the supernatant was collected. Total protein concentrations in the supernatants were then calculated spectrophotometrically using a NanoDrop One (Thermo Fisher Scientific, USA). The protein samples were mixed with sample buffer containing 1.25% 2-mercaptoethanol, and then denatured at 70°*C* for 10 *min* using a dry block heater. The denatured protein samples and a protein standard were loaded onto a Bolt Bis-Tris Precast Gel (4-12% gradient) in XCell SureLock Mini-Cell Electrophoresis System (Invitrogen, USA). The proteins were separated by running the gel at 160 *volts* in the running buffer solution. After electrophoresis, the gels were removed and placed in iBlot® 2 Regular Transfer Stack (IB24001; Invitrogen, USA) and the proteins were transferred to the PVDF membrane in 7 *min* using iBlot 2 Dry Blotting System. iBind solution kit and cards were used and membrane detection was performed with iBind Western Device (SLF1000; Invitrogen, USA). To determine the target protein levels, the PVDF membrane was first blocked and then incubated with the NOX5 primary antibody (Cat. No. ab191010, diluted 1:400; Abcam, UK) followed by the HRP-conjugated secondary antibody. After

detection, the membranes were washed with distilled water and visualized using ECL chemiluminescence kit (K-12045-D20; Advansta, USA). The protein amounts were normalized using β-Actin. The protein band intensities were quantified densitometrically using the ImageJ software, and the numerical data was obtained.

Statistical analysis: Statistical analyses were performed using Prism 5.0 software (GraphPad, USA). The results were presented as mean± standard deviation for descriptive statistics. Comparisons between independent sample groups were made using the Student's t-test. A $p<0.05$ value was considered statistically significant (*p< 0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

Results

Morphological analysis: Morphological evaluation of sperm cells of NS and OAS groups was performed by Spermac staining and Diff-quick staining methods. While sperm with normal morphology were observed in NS patients, defects such as neck fracture, curled tail, and cytoplasmic droplets were observed in OAS patients (Figures 1 and 2).

Oxidant and antioxidant levels: When the groups were compared in terms of TOC, the oxidant level in the OAS group (60.43±20.53 *mg/L*) was found to be higher than in the NS group (32.45±11.59 *mg/L*), and this difference was statistically significant (p<0.001). When the groups were compared in terms of TAC, higher antioxidant levels were determined in the NS group (1.16±0.19 *mg/L*) than in the OAS group (0.98±0.26 *mg/*.) (p<0.05). The oxidative stress index increased significantly in the OAS group (5.60±2.49) compared to the NS group (3.36±1.57) (p<0.001) (Figure 3).

Spermchromatin integrity: When the NS and OAS groups were compared in terms of sperm chromatin integrity, the number of sperm stained darker

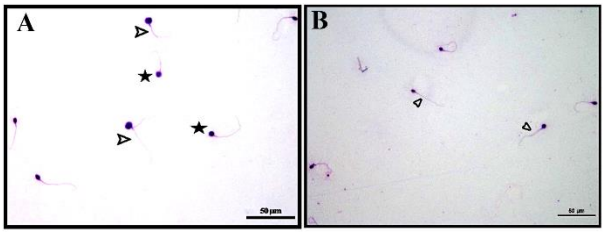

Figure 1. Diff-quick staings. Sperm cells with double tail anomaly (white arrowhead) and sperm cells with round head anomaly (star) belonging to the OAS group stained with diff-quick dye are shown (A). Sperm cells (white arrowhead) belonging to the NS group are shown (B). Bar=50 *μm*

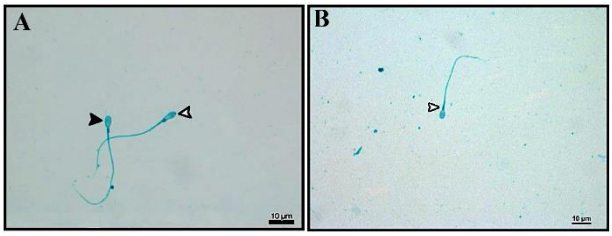

Figure 2. Spermac staining. Morphologies of sperm cells stained with spermac stain are shown. Cells with normal head morphology (black arrow) and cells with long head morphology (white arrow) in the OAS group are shown (A). Sperm cells belonging to the NS group are shown (B). Bar=10 *μm*

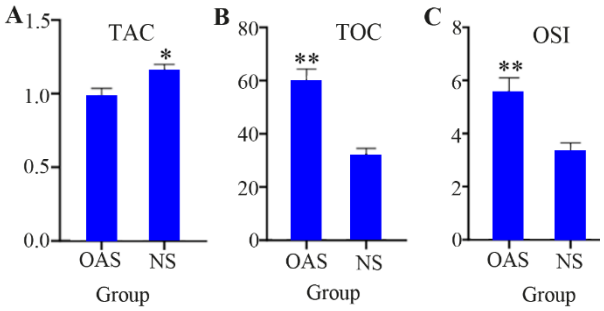

Figure 3. Oxidant and antioxidant amounts between the OAS and NS groups. Values are mean±standard deviation. (*p<0.05; **p<0.001). (A, B, C) They show TAC (*mg/L*), TOC (*mg/L*) and OSI levels between groups

in the OAS group (74.88±10.41%) was higher than the number of sperm in the NS group (24.16±7.70%), and this difference was statistically significant (p<0.001) (Figure 4).

Immunohistochemical staining analyses: While staining was observed in the sperm plasma membra ne and tail in the OAS group, no staining was observed in the NS group. When the OAS and NS group were compared in terms of expression rates, the OAS group showed statistically significant increases in NOX5 protein expression in the sperm plasma membrane (OAS: 61.96±17.92%; NS: 14.17±9.31%) (p<0.001), tail (OAS: 45.30± 17.57%; NS: 9.47±7.66%) (p<0.001), and plasma membrane plus tail (OAS: 32.04±16.25%; NS: 7.63±6.45%) (p<0.001) sections (Figure 5, Table 1).

NOX5 expression correlated positively with chromatin integrity and oxidative stress (p<0.01). In addition, as NOX5 expression increased, the motility rate decreased, indicating a negative correlation between the two parameters (p<0.01) (Table 2).

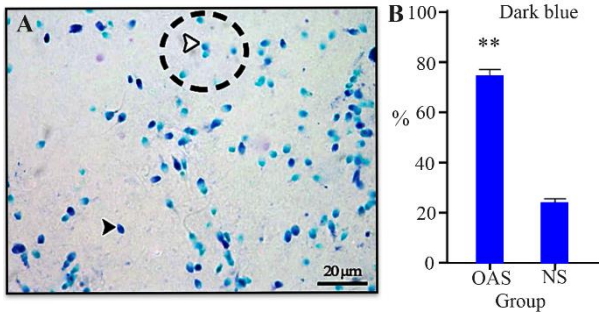

Figure 4. Toluidin Blue staining. A) Abnormal chromatin integrity were statined as dark blue, black arrow shows TB (+). Normal chromatins stained as light blus and white arrow shows TB (-). Bar: 20 *µm*. B) Chromatin integrity comparison between OAS and NS groups. Values are mean±standard deviation (**p<0.001)

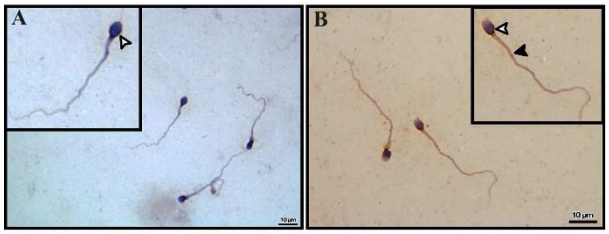

Figure 5. NOX5 immunohistochemical staining in sperm. A) White arrow head shows sperm plasma staining in NS. B) Plasma membrane (white arrow) and tail (black arrow) NOX5 staining in OAS. Bar=10 *µm*

Western blot analyses: When the OAS and NS groups were compared, the amount of NOX5 protein expression in the OAS group (1.15±0.05) was significantly higher than in the NS group (0.83± 0.03) (p<0.002). These western blot results confirm the findings from immunohistochemistry analysis (Figure 6).

Table 1. Evaluation of immunohistochemical scoring results of NOX5 protein in OAS and NS groups

Group		Mean ± S.D	p-value
Plasma membrane (%)	OAS	61.96±17.92	p<0.001
	NS	14.17±9.31	
Tail (%)	OAS	45.30±17.57	p<0.001
	NS	9.47±7.66	
Plasma membrane + tail (%)	OAS	32.04±16.25	p<0.001
	NS	7.63±6.45	

NOX5 protein localization in sperm cells of OAS and NS groups was determined by immunohistochemistry. In the statistical evaluation of the staining ratios of OAS and NS groups, it was observed that NOX5 protein was significantly increased in the plasma membrane (P.M.) (p<0.001), tail (p<0.001) and plasma membrane+tail (P.M.+tail) (p< 0.001) parts of the sperm (p<0.001)

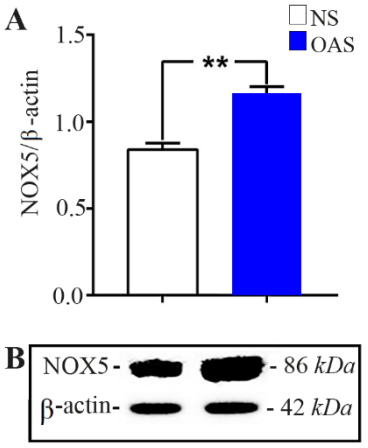

Figure 6. NOX5 protein expression level comparison between NS and OAS. A) NOX5 protein were significantly higher in OAS group. B) NOX5 protein. Values are mean ± standard deviation (**p<0.001)

Table 2. NOX5 expression levels correlations with the chromatin structure, oxidative stress and motility in OAS group (P.M: Plasma Membrane; O.S. Oxidative Stress)

Variables	Plasma membrane	Tail	P.M. + Tail	O.S.	Chromatin structure	Motility
Plasma membrane	-	R=0.94 p<0.01	R=0.90 p<0.01	R=0.58 p<0.01	R=0.84 p<0.01	R=-0.64 p<0.01
Tail	R=0.94 p<0.01	-	R=0.95 p<0.01	R=0.47 p<0.01	R=0.82 p<0.01	R=-0.62 p<0.01
P.M + Tail	R= 0.90 p<0.01	R=0.95 p<0.01	-	R=0.47 p<0.01	R=0.74 p<0.01	R=-0.54 p<0.01
O.S.	R=0.5 p<0.01	R=0.47 p<0.01	R=0.47 p<0.01	-	R=0.47 p<0.01	R=-0.27 p<0.05
Chromatin structure	R=0.84 p<0.01	R=0.82 p<0.01	R=0.74 p<0.01	R=0.47 p<0.01	-	R=-0.73 p<0.01
Motility	R=-0.64 p<0.01	R=-0.62 p<0.01	R=-0.54 p<0.01	R=-0.27 p<0.05	R=-0.72 p<0.01	-

Immunohistochemistry was used to find out where the NOX5 protein was located in sperm cells from the OAS and NS groups. Determination of oxidative stress in the groups was performed by TAC and TOC. Toluidine blue staining was performed to determine the sperm chromatin structure in the groups

Discussion

In the present study, the expression of NOX5 protein, which is reported to be a candidate for ROS production in sperm, was investigated in semen samples of normozoospermic and oligo-asthenozoospermic men, and its effects on chromatin integrity, oxidative stress levels, and sperm motility were investigated. Previous studies have clearly demonstrated that NOX5 is expressed in the uterus, spleen, lymph nodes, testis, endothelial cells, pachytene spermatocytes, and round spermatids, and is involved in the edox-active systems (13, 16). These studies were conducted not only in human spermatozoa but also in equine and dog spermatozoa (14, 15). Sabeur and Ball (2007), in a study investigating the characterization of NOX5 in equine testis and spermatozoa, reported that NOX5 is present in equine spermatozoa and testicles and represents a potential mechanism for ROS generation in equine spermatozoa (15). In addition, Musset et al. (2012) investigated NOX5 expression in human spermatozoa and reported that NOX5 is an important source of ROS in human spermatozoa and plays a role in NOX5-dependent ROS formation related to human sperm motility (29). These results show that NOX5 is the main source of ROS generation in human spermatozoa. Vatannejad et al. (2019) evaluated NOX5 expression and oxidative stress in spermatozoa cells from normozoospermic and asthenozoospermic men and reported that overexpression of NOX5 can give rise to extreme ROS production and oxidative stress damage to DNA and plasma membrane integrity in asthenozoospermic men (30). In studies investigating the expression, function, and regulation of NOX5 in human spermatozoa, it has been reported that NOX5 is expressed in human spermatozoa and produces superoxide (29). In addition, in a study investigating the role of NOX5 in the effect of calcium (Ca^{+2}) and ROS on human sperm function, it was reported that inhibition of NOX5 activity decreases sperm motility (29, 31). NOX5 is the only isoform with calcium binding sites, and calcium is crucial for NOX5 activation. Ca^{+2} entry provides a potential mechanism to link NOX5 activation and ROS-induced hypermotility, thus reinforcing the concept that NOX5 has a central role in sperm motility. The findings of our study also showed an increase in the amount of ROS in the OAS group, which correlated with the incraased amount of NOX5 expression, and this increase in ROS level caused oxidative stress. On the other hand, it was determined that there was a decrease in sperm motility in the OAS group compared to the NS group. The findings presented in this study are consistent with previous research that has demonstrated elevated levels of ROS generation and reduced motility in abnormal spermatozoa compared to normal spermatozoa within a given population (32-34).

In the study investigating the expression of NOX5 in human semen samples from normozoospermic and teratozoospermic groups, Ghani et al. (2013) conducted immunofluorescence staining to observe the presence of NOX5 in various regions of sperm, including the acrosomal, post-acrosomal, equatorial, body, and tail regions. They reported that higher NOX5-positive sperm cells and greater NOX5 expression were observed in the teratozoospermic group compared to the normozoospermic group. Moreover, they reported that positive correlations were observed between abnormal sperm morphology and both the percentage of NOX5-positive sperm and the magnitude of NOX5 expression. As a result of these findings, they suggested that there is a positive correlation between ROS production and OX5 expression in cases with teratozoospermia (28). In the present study, in which the expression of NOX5 in sperm of the NS and OAS groups was investigated by immunohistochemical staining, NOX5 protein was highly expressed in the sperm of the OAS group, particularly in the plasma membrane and tail regions of sperm. In addition, western blot analyses were performed to confirm the accuracy of immunohistochemistry results. Based on the findings, higher NOX5 protein expression was observed in the OAS group. In this context, the results of the present study are consistent with the previous findings reported in the literature. Given that NOX5 expression and levels were observed to be high in the OAS group, these findings suggest that increased NOX5 may contribute to male infertility.

Vatannejad et al. (2019) evaluated NOX5 expression and oxidative stress in human spermatozoa cells obtained from normozoospermic and asthenozoospermic men and reported that chromatin structure deteriorates as a result of increased NOX5 protein expression (30). In our study, the chromatin structure in OAS and NS groups was analyzed with toluidine blue staining. Toluidine blue, which is a cationic dye, binds to phosphate groups in sperm DNA and can be used to detect the deterioration in sperm chromatin

structure. In the current study, the chromatin integrity structure between the study groups was compared and the relationship with other investigated parameters, such as NOX5 expression, oxidative stress, and sperm motility was investigated. As a result of the evaluations, it was found that increased expression of NOX5 protein was positively correlated with oxidative stress and chromatin structure and negatively correlated with motility. Although the production of ROS by spermatozoa cells is associated with low fertility, it is known that a certain level of redox activity is physiologically necessary for the normal functions of sperm such as the acrosome reaction, hyperactive motility, and capacitation (35). Numerous studies have shown a relationship between spermatozoon quality and chromatin integrity. Previous research indicated a positive correlation between TAC, progressive motility, and normal morphology in human spermatozoa (36). In another study, while increased oxidative stress was observed in asthenozoospermic patients, the increased amount of ROS suppressed the antioxidant defence mechanism and negatively affected sperm function by causing plasma membrane and DNA damage. In addition, the increased amount of oxidative stress led to impaired sperm motility (30). Pourmasumi et al. (2019) evaluated sperm chromatin integrity using toluidine blue (TB) and aniline blue (AB) staining in infertile and normozoospermic men, and observed a negative correlation between sperm chromatin structure, sperm count, normal morphology, progressive motility, and sperm chromatin condensation. They suggested that AB and TB staining tests may be useful in evaluating male fertility potential (37). The results of our present work are not compatible with the aforementioned study. In the study by Pourmasumi et al., the infertile men had idiopathic in-fertility, meaning the exact etiology was un-known. Given the lack of a clear underlying cause, these men may have presented with various sperm abnormalities, such as oligoasthenospermia, as thenospermia, and oligospermia. In contrast, the current study specifically included pa-tients with oligoasthenospermia.

Advancements in understanding the biochemistry and biology of NOX5, as well as the identification of mechanisms by which increased ROS impact male reproductive function, will help to further elucidate the role of NOX5 in human health and disease. The results from this study revealed that NOX5 is present in men with oligoasthenospermia, and that ROS-dependent NOX5 protein is associated with sperm chromatin structure, oxidative stress, and impared sperm motility in male infertility. These findings suggest a potential mechanism underlying ROS production in these cases.

Conclusion

These results suggest that overexpression of NOX5 may be the source of excessive ROS production and oxidative stress injuries in oligoasthenozoospermic men. These results show that NOX5 has a significant effect on male infertility. Considering that NOX5 expression is positively correlated with oxidative stress and chromatin integrity, but negatively correlated with motility, NOX5 may serve as a useful biomarker when evaluating samples for assisted reproductive procedures. Further experimental and clinical studies are needed to understand the regulatory roles of NOX5 in pathological processes in male infertility.

Acknowledgement

None.

Funding: This research did not receive any specific grants from funding agencies.

Conflict of Interest

The authors declared that there is no conflict of interest.

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