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Biochemical and Molecular Changes Associated with Asthenozoospermia

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ONE of the primary causes of male infertility is the asthenozoospermia (AZS) which is characterized by defective sperm motility. More genes have been recently associated with AZS. Oxidative stress is also another notable cause for AZS. This study aimed to investigate the biochemical (oxidant/antioxidant status) and molecular (AZS-associated genes) changes in asthenozoospermic patients. To achieve this goal, we utilized spectrophotometry and real time PCR (qPCR) assays on semen samples collected from AZS patients (n = 50) and normozoospermia men (n = 25). Semen samples were categorized into these two categories based on the sperm physical characters, motility, viability, and morphological parameters. The examined parameters included the oxidative peroxide malondialdehyde (MDA), the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and asthenozoospermic genes (*CATSPER1, SEPT12, CFAP43,* and *CFAP44*). Semen of AZS patients exhibited significantly (P <0.05) higher MDA levels, significantly (P <0.05) reduced expression of *CATSPER1, SEPT12, CFAP43,* and *CFAP44* and activities of CAT, SOD, and GPx compared to semen of the control (normozoospermia) group. Among the four genes, only *CATSPER1* and *SEPT12* expression showed a significantly (P <0.05) negative correlation with abnormal sperm forms and seminal MDA levels and a significantly positive correlation with sperm motility and count and seminal levels of the antioxidant enzymes. These findings provided empirical proof that decreased *CATSPER1* and *SEPT12* expression and increased seminal oxidative stress are associated with asthenozoospermia.

Keywords: Asthenozoospermia, Male infertility, CATSPER1, SEPT12, Oxidative stress.

Introduction

There is currently no treatment or cure for infertility, despite its prevalence across the globe. It's believed that 8-12% of couples in the reproductive age range are afflicted by infertility, and that both sexes are similarly impacted [1]. About 30% of infertile couples have no underlying reason identified (named idiopathic infertility), demonstrating that our understanding of infertility is far from comprehensive. Asthenozoospermia (AZS), or low sperm motility (below 34% progressive forward motility), is a common male factor in infertility, affecting an estimated 20% of infertile men [2]. The sperm tail (flagella) is driven by ATP, which is synthesized in the mitochondria located in the middle piece of the sperm [3]. Spermatozoa need to be highly motile so that they can pass through the female reproductive system till reaching the oocytes. Two types of sperm motility

can be recognized in normozoospermic (i.e. normal motile sperms) individuals: activated motility occurred in the ejaculated semen, and hyperactivated motility detected near oocytes at the fertilization site and is necessary for sperm capacitation and the acrosome reaction [4]. Multiple metabolic pathways and regulatory mechanisms involved in the normal motility of spermatozoa. Furthermore, poor sperm motility and consequent sterility may be attributable to the particular gene deficiency association and any abnormalities of these components [4].

There has been a lot of research done recently on the part oxidative stress (OS) plays in the etiology of male infertility. Reactive oxygen species (ROS) and other free radicals are produced in excess in people with OS, and reduced antioxidant defences. About 35% of infertile men have higher OS in their semen, and increased levels

of seminal ROS have been linked to a variety of diseases affecting the male reproductive system [5]. The male germ cell is especially susceptible to excessive levels of ROS due to its distinctive design, which includes an abundance of oxidizable substrates and inadequate internal antioxidant defenses. These OS then contribute to the malfunction and eventual apoptosis of germ cells [6]. As a result, low sperm motility is often seen in individuals with elevated seminal ROS levels [7]. Indeed, studies of asthenozoospermic people consistently report higher levels of seminal ROS [8-10]. Asthenozoospermia is often caused by OS-induced dysregulation of motility-associated signaling pathways [11].

A number of genes, such as *CATSPER1, SEPT12, CFAP43,* and *CFAP44*, have been associated with asthenozoospermia [1,4,12,13]. CATSPER1 expression is localized to the sperm plasma membrane just above the fibrous sheath in the principal tail piece and plays a crucial role in the induction of sperm hyperactivity-dependant capacitation through opening flagellum Ca^{2+} channels during passing through female genital tract especially near the fertilization site [12,13]. Therefore, lack of this protein inhibits sperm capacitation thereby leading to asthenozoospermia [14]. Moreover, SEPT12 is one of the main 5 septin proteins that form the annulus, a terminal ring of protein localized between the midpiece and the principle piece of the spermatozoon [15]. Lack of annulus formation due to decreased septin proteins, especially SEPT4 and SEPT12, causes an aberrant flagellum and sperm motility [16,17]. On the other hand, cilia and flagella-associated protein (CFAP) producing genes *CFAP43* and *CFAP44* also play important roles in sperm motility and their mutations associated with lack of movement and asthenozoospermia [18].

Little is known regarding the correlation between the expression levels of AZS-related genes (*CATSPER1, SEPT12, CFAP43,* and *CFAP44*) and the semen quality parameters and oxidant/ antioxidant status in semen of AZS patients. Therefore, this study was conducted to investigate this correlation.

Material and Methods

Sampling and Semen Analysis

A total of 75 semen samples were collected from 75 patients (ages between 29 and 41 years) at a private center for male infertility in Mansoura, Egypt.[We got an ethical approval (KFS-IACUC/113/2021) from Kafrelsheikh University Ethical Committee] Samples were collected by masturbation after 3 – 5 days of sexual abstinence. All patients signed informed consents before taking seminal samples. Semen samples were examined microscopically to determine sperm count (10^6/ ejaculate), progressive motility (%), viability (%), and morphology (% abnormal forms) by computer-assisted sperm analyzing (CASA) system using protocols and following guidelines of World Health Organization (WHO) (2010). We excluded any abnormal samples such as highly viscous samples.

Experimental Design

The samples were divided into two groups: the asthenozoospermic (n = 25) and normozoospermic (n=25) group. The samples were classified according to their progressive motility, which is the ability of sperm to move forward in a straight line. Samples with progressive motility below 32% were categorized as asthenozoospermic, meaning they had low sperm motility. Samples with progressive motility above 32% were categorized as normozoospermic, meaning they had normal sperm motility.

Determination of MDA Levels in Semen

The level of lipid peroxidation was measured by the amount of malondialdehyde (MDA), a by-product of lipid breakdown using commercially available kit (Biodiagnostic, Cairo, Egypt) and as previously described [19,20]. MDA reacted with thiobarbituric acid (TBA) in an acidic solution, forming a red-colored compound. The intensity of the red color was measured by a spectrophotometer.

Determination of Antioxidant Activities in Semen

The activities of the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined in semen samples following manufacturer protocols (Biodiagnostic, Cairo, Egypt) and in accordance with our previous description [19,21]. SOD prevents the change of epinephrine to adrenochrome at a pH of 10.2, which makes it possible to measure its concentration [22]. The speed of H_2O_2 breaking down at 240 nm was used to calculate CAT activity [23].

Real time PCR (qPCR)

We used qPCR to measure the expression of genes related to asthenozoospermia (*CATSPER1, SEPT12, CFAP43,* and *CFAP44*) in semen samples. We extracted total RNA from semen samples with Trizol (Invitrogen). Then, we converted the RNA to cDNA with reverse transcriptase (RevertAid H Minus Reverse kit, Thermo Scientific) and measured the cDNA with a Nanodrop. We performed the qPCR with a Piko qPCR thermal cycler (Thermo Scientific) and analyzed the data with its software. We added Syber Green (Thermo Scientific) to the PCR mixture with cDNA and primers. We followed the manufacturer's recommended temperature range as described before [24-26]. We used the $2^{-\Delta\Delta Ct}$ method to

calculate the gene expression change relative to the *B-actin* gene.

Statistical Analysis

GraphPad Prism 8.0 (San Diego, CA) was utilized to analyze the data statistically. We compared the groups with the student t test. The strength of the relationships between the variables was measured using Pearson's correlation coefficient. The significance level was set at P <0.05, and data were reported as mean±SEM.

Results

Semen analysis of NZS and AZS patients

The semen quality parameters, including total and forward progressive motility, sperm count and abnormal sperm forms, in the normozoospermic (NZS) and asthenozoospermic (AZP) groups are shown in Figure 1. All semen quality parameters were significantly (p ≤ 0.05) lower in the AZS group than the NZS group, except the morphology which denoted significantly (p ≤ 0.05) higher % of the abnormal sperm forms in the AZS group than the NZS group.

Oxidant and Antioxidant Status in NZS and AZS semen

Figure 2 compares oxidative (MDA) and antioxidant (SOD, Cat, and GPx) status in NZS and AZS semen. Semen of the AZS patients had significantly (P<0.05) elevated MDA levels and significantly (P<0.05) declined activity of SOD, CAT, and GPx enzymes, with lowest levels for SOD followed by Cat then GPx, compared to the NZS group.

Molecular Analysis of NZS and AZS Semen

Figure 3 illustrates the relative expression of genes related to asthenozoospermia (CATSPER1, SEPT12, CFAP43, and CFAP44) in semen samples. Semen of AZS patients exhibited significantly (p ≤ 0.05) downregulated expression of CATSPER1, SEPT12, CFAP43, and CFAP44 relative to semen of the NZS group. Comparing the four genes, SEPT12, CFAP43 expression was lower than CFAP43, and CFAP44 in the AZS group.

Correlation Between Gene Expression and Semen Quality Parameters

Table 1 shows the correlation between the expression of four genes related to asthenozoospermia (CATSPER1, SEPT12, CFAP43, and CFAP44) in the semen of AZS patients and their sperm parameters. The gene expression was positively correlated with total sperm motility (r = 0.096 – 0.210), progressive sperm motility (r = 0.210 – 0.437), and sperm count (r = 0.024 – 0.158). The gene expression was negatively correlated with abnormal sperm forms (r = - 0.523 - 0.142). Only the CATSPER1 and SEPT12 genes showed significant (P<0.05) correlations, both positive and negative, with the sperm parameters.

Correlation Between Gene Expression and Oxidant/Antioxidant Status

As shown in Table 2 expression of CATSPER1, SEPT12, CFAP43, and CFAP44 genes in the semen of AZS patients was negatively correlated with seminal MDA levels (r = - 0.190 – 0.366) and positively correlated with seminal levels of the antioxidant enzymes SOD, CAT and GPX (r = 0.329 – 0.650). Again, only the CATSPER1 and SEPT12 genes showed significant (P<0.05) correlations, both positive and negative, with the oxidant and antioxidant parameters, respectively.

Discussion

About 40% of couples who cannot conceive have a male partner with infertility, which is a global medical and social issue [2]. Asthenozoospermia (AZS) is characterized by low sperm motility that may result from problems in the development of the sperm tail or the energy production system that is needed for progressive movement [1]. Some genes that have been linked to AZS are *CATSPER1, SEPT12, CFAP43, and CFAP44* [1,4,12,13]. Little is known regarding the correlation between the expression levels of these genes and both the semen quality parameters and oxidant/antioxidant status in the semen of AZS patients. This prompts us to investigate this correlation. Our data confirmed those obtained by Abd Elrahman*, et al.* [27] and showed that AZS men had lower total and progressive sperm movement and higher abnormal sperm shape than men with normal sperm motility (NZS).

Sperm dysfunction in male infertility can be caused by oxidative stress (OS), which affects the quality and fertilizing ability of sperm. ROS are needed for sperm function in small amounts, but too much can harm the sperm. In agreement, we also found a significantly higher OS (as revealed by decreased seminal MDA levels) in semen of AZS patients compared to the NZS men. Our results agree with many previous studies. High levels of ROS in the semen can cause sperm to move slowly or not at all [7]. People with asthenozoospermia have more ROS in their semen than normal [8-10]. OS can often impair the signaling pathways related to motility, leading to asthenozoospermia [11].

MDA is a marker of oxidative stress that shows lipid peroxidation [28-31], which is the damage of lipids in the sperm membranes by ROS. High MDA levels may mean more ROS production and more oxidative damage in infertile men. This can affect sperm movement and cause other problems for the sperm cells, such as dysfunction and apoptosis [6]. Our result agrees with Abd Elrahman, et al. [27] and Masroor et al. [32], who found higher MDA levels in men with low sperm motility than in men with normal sperm motility. Some studies suggested that low sperm motility may be related to faulty mitochondria and reduced energy production in the sperm cells as a result of OS [33,34]. On the other hand, we found significantly lower activities of the antioxidant enzymes (SOD, CAT, and GPx) in semen of AZS patients than in the NZS men. These enzymes reflect of the activity of the endogenous intracellular antioxidant enzymes in sperms and in the seminal plasma, which can protect the sperm from OS. Consistent with our findings, several other studies reported declined antioxidant activities in AZS men and attributed this negative impact to high ROS production and sperm abnormalities [27,32, 35,36].

In the present study, we found that AZS patients had significantly (p ≤ 0.05) lower CATSPER1, SEPT12, CFAP43, and CFAP44 mRNA levels than the NZS group. Similarly, several other previous studies reported downregulated expression in semen of AZS patients and considered these genes as molecular markers for asthenozoospermia [1,4, 12,13]. Among these four genes, only the *CATSPER1* and *SEPT12* genes showed significant (P<0.05) positive correlation with total sperm motility, progressive sperm motility, sperm count and seminal levels of the antioxidant enzymes SOD, CAT and GPX, but with a significant

(P<0.05) negative correlation with abnormal sperm forms and seminal MDA levels. CATSPER1 is a protein that is found on the sperm membrane in the principal piece of the tail and helps the sperm to become more active and ready to fertilize the oocyte by opening calcium channels in the flagellum when the sperm travels through the female reproductive tract, especially near the site of fertilization [12,13]. Therefore, if this protein is missing, the sperm cannot fertilize the oocyte and causes asthenozoospermia [14]. In addition, SEPT12 is one of the five septin proteins that make up the annulus, a ring of protein that separates the middle and the main segments of the sperm tail [15]. If the annulus is not formed properly due to low levels of septin proteins, especially SEPT4 and SEPT12, the flagellum becomes abnormal and the sperm movement is impaired [16,17]. Furthermore, genes that produce cilia and flagella-associated proteins (CFAP), such as CFAP43 and CFAP44, also have important roles in sperm movement and their mutations are linked to lack of movement and asthenozoospermia [18].

The study had some limitations, such as the small number of participants and the focus on the Egyptian men with low sperm movement. Therefore, it was difficult to verify the impact of changes in the *CATSPER1* and *SEPT12* expression on male infertility. However, the results suggested some scientific signs that some of the cases tested had a link between *CATSPER1* and *SEPT12* expression and asthenozoospermia. More research is needed with more patients, and genes, to help us understand how different gene expression affect the development of asthenozoospermia and other forms of male infertility.
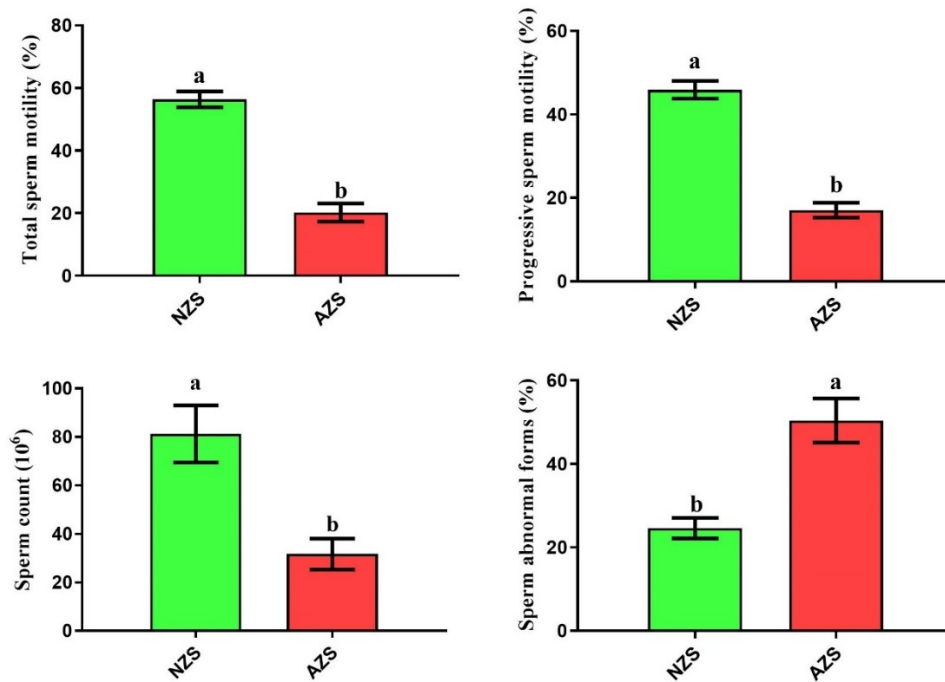
Fig. 1. Semen quality parameters (total and progressive sperm motility, sperm count, sperm abnormal forms) in AZS and NZS men. Data was presented as mean ± SEM. Columns (means) and error bars (SEM) had different letters indicate statistical differences at p < 0.05. NZS, normnozoospermic group and AZS, asthenozoospermic group.

Fig. 2. Semen of the AZS group had higher oxidative stress and lower antioxidant enzymes activities than the NZS group. Data was presented as mean ± SEM. Columns (means) and error bars (SEM) had different letters indicate statistical differences at p < 0.05. NZS, normnozoospermic group and AZS, asthenozoospermic group.
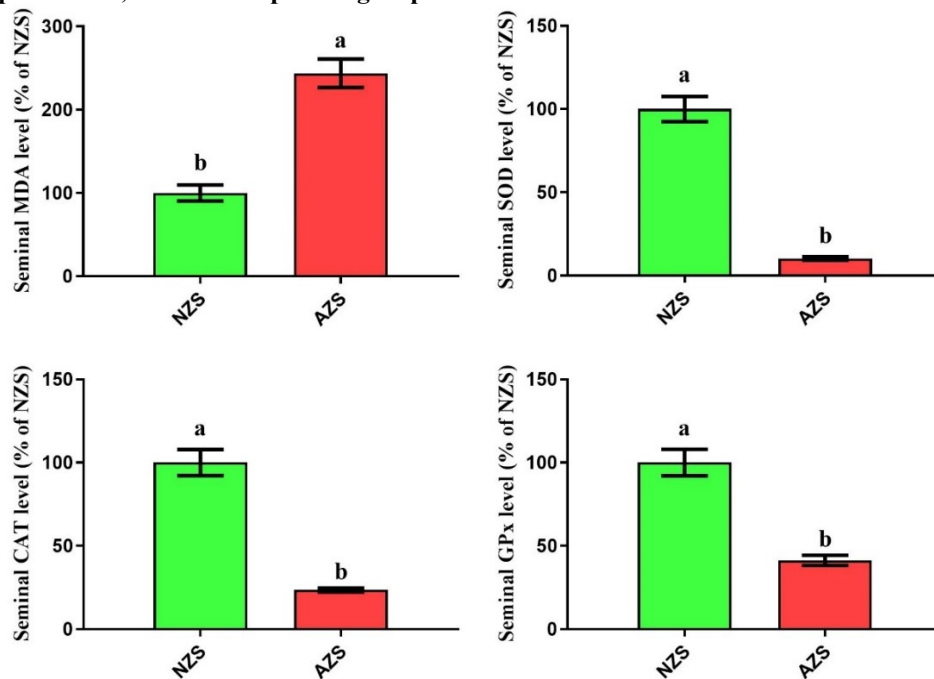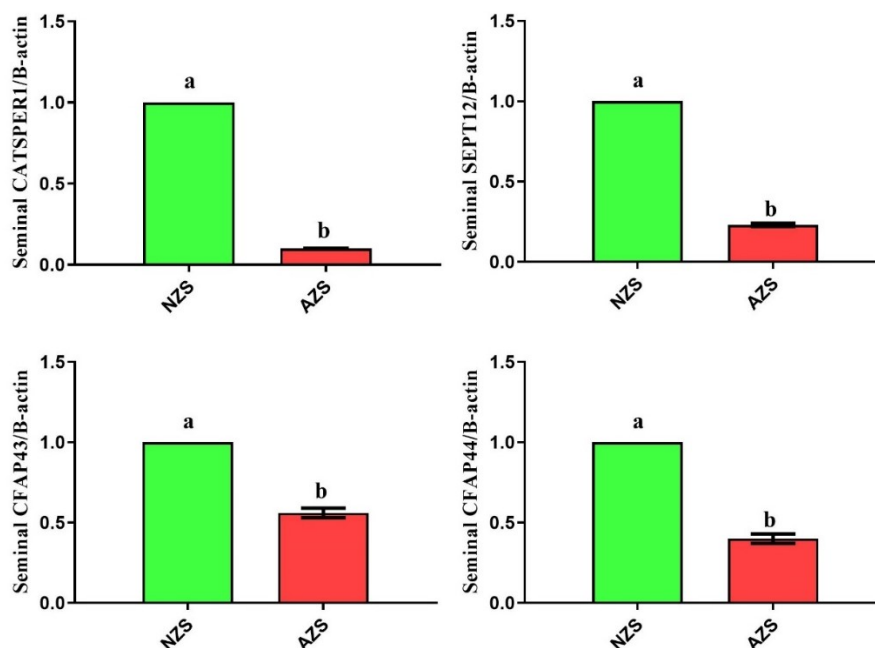
Fig. 3. Altered expression of genes related to asthenozoospermia (*CATSPER1, SEPT12, CFAP43,* and *CFAP44*) in the semen samples of the NZS and AZS groups as detected by qPCR. The relative gene expression was presented as mean ± SEM. Columns (means) and error bars (SEM) had different letters indicate statistical differences at p < 0.05. NZS, normnozoospermic group and AZS, asthenozoospermic group.

TABLE 1. Correlation between gene expression and semen quality parameters.

	Gene expression, r (p value)			
	CATSPER1	**SEPT12**	**CFAP43**	**CFAP44**
Total motility	0.210 (0.032)	0.196 (0.037)	0.096 (0.091)	0.118 (0.085)
Progressive motility	0.437 (0.019)	0.390 (0.028)	0.210 (0.079)	0.264 (0.082)
Sperm count	0.158 (0.011)	0.142 (0.020)	0.024 (0.152)	0.040 (0.127)
Abnormal sperm forms	- 0.142 (0.040)	- 0.176 (0.047)	- 0.523 (0.077)	- 0.487 (0.081)

r, Pearson's correlation

TABLE 2. Correlation between gene expression and oxidant/antioxidant status.

	Gene expression, r (p value)			
	CATSPER1	**CATSPER1**	**CATSPER1**	**CATSPER1**
Seminal MDA levels	- 0.190 (0.010)	- 0.208 (0.021)	- 0.366 (0.119)	- 0.341(0.096)
Seminal SOD levels	0.650 (0.048)	0.500 (0.050)	0.329 (0.090)	0.340 (0.074)
Seminal CAT levels	0.579 (0.027)	0.518 (0.031)	0.370 (0.085)	0.392 (0.079)
Seminal GPx levels	0.522 (0.040)	0.490 (0.044)	0.352 (0.103)	0.364 (0.086)

r, Pearson's correlation

Conclusions

To the best of our knowledge, this is the first study on the Egyptian patients reported that seminal *CATSPER1* and *SEPT12* expression is positively correlated with spermatozoa motility, count and seminal levels of the antioxidant enzymes and negatively correlated with abnormal sperm forms and seminal MDA levels. These results confirmed that downregulated seminal *CATSPER1* and *SEPT12* expression and elevated seminal oxidative stress are associated with asthenozoospermia.

Conflicts of interest

"There are no conflicts to declare".

Funding statement

"There is no funding statement to declare".

Author`s contributions

"Authors contribute equally in this work"

Ethical approve: We got an ethical approval (KFS-IACUC/113/2021) from Kafrelsheikh University Ethical Committee.

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التغيرات البيوكيميائية والجزيئية المرتبطة بضعف حركة الحيوانات المنوية

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أحد الأسباب الرئيسية لعقم الرجال هو ضعف حركة الحيوانات المنوية (AZS) والذي يتميز بعيوب في حركة الحيوانات المنوية. تم ارتباط المزيد من الجينات مؤخرًا بـ AZS. الإجهاد التأكسدي هو أيضًا سبب آخر ملحوظ لـحدوث AZS. هدفت هذه الدراسة إلى استقصاء التغيرات البيوكيميائية (الحالة المؤكسدة و المضادة للأكسدة) والجزيئية للجينات المرتبطة بـ AZS في مرضى ضعف حركة الحيوانات المنوية. لتحقيق هذا الهدف ، استخدمنا اختبارات الطيف الضوئي والتفاعل البوليميراز المتسلسل الحقيقي (qPCR) على عينات السائل المنوي التي تم جمعها من مرضى ضعف حركة الحيوانات المنوية (50 = n) والرجال الطبيعيين (25 = n). تم تصنيف عينات السائل المنوي إلى هاتين الفئتين استنادًا إلى التشخصيات الفيزيائية للحيوانات المنوية ؛ الحركة والحيوية والمعايير المورفولوجية. شملت المعلمات التي تم فحصها البيروكسيد التأكسدي (MDA) ، وإنزيمات المضادة للأكسدة CAT و SOD وGPx ، والجينات المرتبطة بضعف حركة الحيوانات المنوية CATSPER1 و SEPT12 و CFAP43 و CFAP44.أظهر سائل منوي مرضى ضعف حركة الحيوانات المنوية مستويات MDA أعلى بشكل ملحوظ ، وتعبيرًا مخفضًا بشكل ملحوظ لـ CATSPER1 و SEPT12 و CFAP43 و CFAP44 وأنشطة CAT و SOD و GPx مقارنة بسائل منوي المجموعة الضابطة (الطبيعية). من بين الجينات الأربعة ، أظهر تعبير CATSPER1 و SEPT12فقط ارتباطًا سلبيًا ملحوظًا مع الأشكال غير الطبيعية للحيوانات المنوية ومستويات MDA السائلة وارتباطًا إيجابيًا ملحوظًا مع الحركة والعدد الحيواني ومستويات الإنزيمات المضادة للأكسدة. وفرت هذه النتائج دليلاً تجريبيًا على أن انخفاض تعبير CATSPER1 و SEPT12 وزيادة الإجهاد التأكسدي السائلي مرتبطان بضعف حركة الحيوانات المنوية.

الكلمات المفتاحية: ضعف حركة الحيوانات المنوية ، عقم الرجال ، CATSPER1، SEPT12، الإجهاد التأكسدي.