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Clinical use of redox biomarkers for diagnosis of male infertility

Marek Szymanski[1–3], Radoslaw Janicki[2, 3], Piotr Kaminski[4], Karolina Wasilow[2], Jaromir Soltysiak[5], Robert Szyca[6], Angelika Szymanska[7], Marcin Arabik[8]

[1]Department of Obstetrics and Gynecology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland

[2]NZOZ Medical Center, Clinic of Infertility Treatment "Genesis", Bydgoszcz, Poland

[3]Gynecology and Oncological Gynecology Ward, 10th Military Research Hospital and Polyclinic, IPHC, Bydgoszcz, Poland

[4]Department of Ecology and Environmental Protection, Department of Medical Biology and Biochemistry, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Bydgoszcz, Poland

[5]"Solmed Medical" Center Chojnice, Poland

[6]Clinic of Surgery and Oncological Surgery, 10th Military Research Hospital and Polyclinic, IPHC, Bydgoszcz, Poland

[7]The National Institute of Medicine of the Ministry of Interior and Administration, Warsaw, Poland

[8]Gynecology, Obstetrics and Pregnancy Pathology Department, Multi-speciality City Hospital, Bydgoszcz, Poland

ABSTRACT

Objectives: The aim of the study was to analyze the activity of antioxidant enzymes (glutathione reductase, catalase, superoxide dismutase) and malondialdehyde (MDA) levels in a population of men with abnormal semen parameters and in a population of men diagnosed with normozoospermia.

Material and methods: This study was performed using data collected at the Infertility Treatment Clinic ‚Genesis', Bydgoszcz, Poland, between 1 January 2011 and 31 December 2017. A total number of 455 men meeting the inclusion criteria were selected and divided into the control group (234 men) and the infertility group (221 men). The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) were measured using ready-made kits; lipid peroxidation intensity was determined by the thiobarbituric acid method.

Results: No statistically significant differences were found for activity of SOD, GR, CAT between the groups. MDA values measured in the serum of patients in the healthy group were higher than in the group with semenological disorders.

Conclusions: Although our study did not demonstrate the usefulness of the above blood tests, further studies are needed to explore the potential use of assessing redox parameters to develop new diagnostic and therapeutic approaches for male infertility.

Keywords: male infertility; oxidative stress; reactive oxygen species; superoxide dismutase; catalase; glutathione reductase; MDA; antioxidant defense

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INTRODUCTION

According to the World Health Organization [1], infertility refers to the biological inability of an individual to achieve pregnancy following at least 12 months of unprotected intercourse [2, 3]. It has been estimated that approximately 15% of couples face some form of infertility and among them, male factor infertility plays a role in nearly 30–50% of all infertile couples [4, 5]. Male infertility diagnosis is commonly based on standard semen parameters analysis [6], according to the WHO guidelines, nevertheless, a large proportion of infertile males does not receive a clear diagnosis, considering them as idiopathic or unexplained cases [7, 8].

Epidemiological predictions indicate that the number of infertile couples will increase. In developed countries, the problem of primary infertility is more frequent, while

Corresponding author:
Marcin Arabik
Gynecology, Obstetrics and Pregnancy Pathology Department, Multi-speciality City Hospital, Bydgoszcz, Poland
e-mail: lekarz.arabik@gmail.com

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developing countries, more often face the problem of secondary infertility. The infertility rate is not the same in each country, and it is determined by many factors, including civilization factors. In developed countries, it ranges between 10% and 12% [1].

According to forecasts, Poland's population will shrink by more than 10% over the next three decades, and such a drastic decline in population will be mainly due to declining birth rates. Obviously, this is influenced not only by the biological ability to reproduce, but also by the desire for parenthood or economic considerations. Nevertheless, making it possible to satisfy the need for an offspring through proper prevention, diagnosis and treatment of infertility should be one of the priorities of modern medicine, not only for the sake of future parents, but for the entire population. The modern model of life promoting the delay of procreative plans, fraught with a great deal of stress, as well as increasingly unfavorable environmental conditions, exacerbate the problem of infertility, posing a very great challenge to reproductive medicine [9].

Diagnosis of the causes of infertility should be carried out simultaneously in both partners. Male fertility is a direct result of the process of spermatogenesis, which involves the continuous production of sperm by the spermatogenic epithelium. It occurs in the spermatogenic tubules of the male gonad and lasts about 74 days [10]. It is known that a huge role in the regulation of physiological processes, including spermatogenesis, is played by health status, *i.e.*, the co-occurrence of conditions both related to the genitourinary system and other systemic dysfunctions. In recent years, the results of numerous studies have repeatedly confirmed the existence of a close relationship between male infertility and their general health status. The occurrence of certain conditions may be related to genetic and/or environmental factors [3, 11–14].

Oxidative stress

Every healthy organism has complex mechanisms whose role is to detoxify reactive oxygen species (ROS). An imbalance between ROS and the body's antioxidant mechanisms can result in sperm damage and loss of conceptus by the negative effects of ROS on sperm function and metabolism, mainly in affecting the processes of capacitation and the acrosomal reaction. During chronic oxidative stress, there is an increase in the amount of oxidized proteins in male gametes, which leads to changes in the structure of the membrane proteins of the sperm head and vitellum. Excessive exposure to ROS significantly reduces male fertility due to damage to sperm cell membranes [3].

Due to their high reactivity, oxygen free radicals, produced in excess, can have a very negative effect on the body. The consequence of this process, when antioxidant defense fails and physiological concentrations of ROS are exceeded, is a condition called "oxidative stress" [15, 16]. Oxidative stress is considered a major etiology for male infertility, more specifically idiopathic infertility. Large proportion of infertile males does not receive a clear diagnosis, considering them as idiopathic or unexplained cases [7].

The analysis of semen parameters according to the WHO guidelines represents, currently, the gold standard for male infertility diagnosis. Several studies showed that ROS-induced sperm oxidation can result in sperm quality alterations, leading to a decrease in sperm fertilizing potential [17–19].

The adverse effects of oxidative stress on sperm function represent a new direction of research into the mechanisms responsible for male infertility [20–22]. Based on the findings presented in the literature, the need to develop new diagnostic methods for male infertility was observed. Along with the assessment of oxidative stress in a seminal fluid, monitoring the redox status of blood could provide a new potential and less invasive practice for clinicians to assess the ability to conception [8]. The redox parameters studied could be useful for developing new therapeutic strategies based on antioxidant supplementation to reduce systemic oxidative stress in patients with infertility, improving the diagnostic process and possible treatment of male infertility and ultimately the success of assisted reproductive technology (ART) [8].

Assays for oxidative stress detection may suggest new biochemical approaches to improve male infertility diagnosis and management, using simple, fast and less expensive techniques [17].

The aim of the study was to analyze the activity of antioxidant enzymes (glutathione reductase, catalase, superoxide dismutase) and malondialdehyde levels in the population of men with abnormal semen parameters and in men diagnosed with normozoospermia and evaluating their usefulness of these determinations in the diagnosis of male infertility.

Atioxidant defense

Both enzymatic and non-enzymatic components of antioxidant defense are involved, with non-enzymatic mechanisms considered complementary elements, while antioxidant enzymes play a major role in the overall process. The most important antioxidant enzymes include superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT). These enzymes are interrelated, cooperating to directly neutralize free radicals, reactivate non-enzymatic components of antioxidant defense, inhibit lipid peroxidation, and repair damaged molecules and destroy those that could not be repaired [23].

Superoxide dismutase (SOD)

Superoxide dismutase is the body's main defense mechanism against the toxic effects of peroxides. It catalyzes the breakdown of superoxide anions to hydrogen peroxide and molecular oxygen. Mitochondria, whose DNA is highly susceptible to free radical attack, must be protected by an efficient superoxide dismutase mechanism. Disruption of the activity of this enzyme can expose the cell to increased attack by ROS, which can damage genetic material, leading to mutations [24].

Catalase (CAT)

Catalase is the main line of defense against highly reactive hydrogen peroxide and is involved in its breakdown into water and oxygen. The enzyme exhibits CAT activity at high concentrations of hydrogen peroxide, causing its breakdown. In contrast, at low concentrations of H2O2, CAT exhibits peroxidase activity, participating in the oxidation of compounds such as methanol, ethanol, formates, nitrites and quinones.

Catalase, by converting hydrogen peroxide, does not generate additional free radicals, which protects cells from other reactive oxygen species. Oxygen from the breakdown of H2O2 can be further utilized in other metabolic processes [24]. This enzyme, by breaking down hydrogen peroxide, reduces the intensity of oxidative stress, and has a compensatory effect against oxidative damage.

Catalase, an enzyme that protects cells from the toxic effects of hydrogen peroxide, has been implicated in mutagenesis, carcinogenesis, inflammation and protection from apoptosis. It is thought that the enzyme's activity may be reduced by prolonged exposure of patients' cells to oxidative stress.

Glutathione reductase (GR)

Glutathione reductase is an enzyme whose role is to restore oxidized glutathione (GSSG) to its reduced form (glutathione regeneration), using electrons derived from NADPH. The reaction catalyzed by reductase proceeds in a sequential census or ping-pong method, which is influenced by the concentration of the oxidized form of glutathione and NADP+. By catalyzing the GSH/GSSG cycle, glutathione reductase prevents excessive accumulation of reactive oxygen species and the formation of associated oxidative damage. This is related to the fact that glutathione disulfide - formed in the reaction catalyzed by glutathione peroxidase — is a cell-damaging compound (capable of inactivating cellular proteins), while it is glutathione in its reduced form that shows antioxidant potential and reacts with hydrogen peroxide [25].

Malondialdehyde (MDA)

Reactive oxygen species are involved in the free radical oxidation of unsaturated fatty acids in lipids, the so-called lipid peroxidation [26]. The end products of lipid peroxidation can be low molecular weight, three-carbon malondialdehyde (MDA) and other aldehydes and hydroxyaldehydes. Malondialdehyde is the most important stable product of lipid peroxidation. Malondialdehyde is one of the most mutagenic products of lipid peroxidation. It reacts with DNA to form premutagenic lesions [27]. Numerous studies show that MDA is one of the primary factors informing about the ongoing processes of lipid peroxidation, and thus indicating the intensification of oxidative stress.

Elevated levels of free radicals intensify lipid peroxidation and increase MDA production. It is believed that concentration of malondialdehyde may be an indicator of increased oxidative stress and antioxidant status of the body [28].

MATERIAL AND METHODS

Research was undertaken following the Guidelines of the European Union Council and the current laws in Poland, according to the Bioethical Commission of the Collegium Medicum of Nicolaus Copernicus University in Torun, Poland. Samples were collected under permit KB 674/2010; No. KB 427/2014 and No. KB 365/2015.

Semen analysis

We conducted semen testing from 2011 to 2017 at the NZOZ Medical Center - Infertility Treatment Clinic 'Genesis', a center accredited by the Polish Ministry of Health as a Medically Assisted Reproductive Center, Cell and Embryo Bank.

Semen testing was performed on each patient-participant in the study. Prerequisites included a 2–7.day period of sexual abstinence. During semen evaluation, macroscopic evaluation was performed, with determination of volume (mL) and reaction (pH) of semen, microscopic evaluation, with determination of concentration (million/mL), motility (type A fast progressive movement (%); type B slow progressive movement (%); type C non-progressive movement (%); type D no movement (%); type A + B progressive movement, A + B + C total movement) evaluation was performed in a Makler chamber and using a light microscope. The morphology (%) of spermatozoa in the semen was also evaluated.

In the semenological analysis, the ejaculate was treated as normal according to WHO 2010 criteria. Based on the results of the standard semen evaluation, the study participants were divided into two groups:

— the control group consisted of 162 patients in whom there were no abnormalities in the analyzed semen

parameters (semen volume, concentration of sperm in semen, motility and morphological structure of sperm) — normozoospermia (WHO 2010);

— the infertility group consisted of 159 men with semen abnormalities applied to oligozoospermia, asthenozoospermia, azoospermia, teratozoospermia, necrozoospermia, combined oligozoospermia- asthenozoospermia-teratozoospermia OAT II, OAT III, cryptozoospermia, polyzoospermia, cryptoteratozoospermia, leukospermia or combined disorders.

Antioxidant enzyme activity and lipoperoxidation intensity

In addition to semen, whole blood of about 15 mL and blood serum were collected from patients approx. 1.5 mL. To obtain the serum, the blood was drawn into "clot" type tubes. The serum was obtained by centrifugation at 3500 rpm for 10 minutes. The serum and semen were separated into Eppendorf-type tubes. The material was transported to the laboratory of the Department of Ecology and Environmental Protection, Collegium Medicum of Nicolaus Copernicus University in Torun, Poland. The whole blood was stored at -80 deg C, as was serum. Semen, on the other hand, was stored in liquid nitrogen.

Determination of antioxidant enzyme activity (SOD, CAT, GR), and lipoperoxidation intensity (MDA) in blood serum was carried out using ready-to-use kits from Cayman Chemicals Co. and Wuhan EIAab Science.

Activity of superoxide dismutase

Serum SOD activity was determined using a standardized Superoxide Dismutase Assay Kit (Cayman Chemical Co. Item No. 706002). The analyses were performed on 96-well plates according to the methodology provided by the manufacturer. Two hundred microliters of radical detector solution (tetrazolium salt solution) were added to the samples and 10 µL of standards. The reaction was started by adding 20 µL of xanthine oxidase solution to all wells. The plate was carefully shaken for several seconds to mix the reaction components and incubated on a shaker for 20 min at room temperature. The absorbance was measured at 450 nm using a plate reader (Multiskan RC Version 6.0, Lab systems). The SOD activity in the samples was calculated from the standard curve and expressed in $U \cdot mL^{-1}$.

Glutathione reductase activity

Glutathione reductase activity in serum was tested using Cayman Chemical's off-the-shelf Glutathione Reductase Assay Kit. The assay was based on measuring the efficiency of NADPH oxidation. The oxidation of NADPH to NADP+ carries a decrease in absorbance at 340nm, which is directly proportional to the GR activity in the sample. Assay Buffer was diluted with high purity water to obtain a buffer containing 50 mM potassium phosphate, pH 7.5, with 1 mM EDTA. Sample Buffer after dilution with high-purity water contained 50 mM potassium phosphate, pH 7.5, with 1 mM EDTA and 1 mg* mL-1 BSA. In this form, it was used to dilute the following reagents. Glutathione reductase control containing GR isolated from baker's yeast was diluted with Sample Buffer (10 µL of enzyme + + 990 µL of Sample Buffer) and placed on ice (according to the accompanying instructions, 20 µL of diluted enzyme when added to the well causes a decrease in absorbance at a rate of about 0.04 absorbance units per minute under standard reaction conditions). GR NADPH — supplied as a lyophilized powder was dissolved in high-purity water. GR GSSG (9.5 mM GSSG solution) was ready to use without prior preparation. The assay was performed in a 96-well plate, the scheme of which was analogous to the GPx assay.

Catalase activity

Serum CAT activity was determined using a standardized Catalase Assay Kit (Cayman Chemical Co. Item No. 707002). The analyses were performed on 96-well plates according to the methodology provided by the manufacturer. Assay Buffer (100 µL) and methanol (30 µL) were added to the samples, to the standards, and to 20 µL of bovine liver catalase, which served as a positive control. The reaction was started by adding 20 µL of hydrogen peroxide to all wells. The plate was incubated for 20 min at room temperature. To terminate the reaction, 30 µL of potassium hydroxide was added to samples, standards, and positive controls, followed by 30 µL of chromogen (Purpald). The plate was then incubated on a shaker for 10 min at room temperature. Next, 30 µL of potassium periodate was added to all wells. The plate was incubated on a shaker for 5 min at room temperature. The absorbance at 540 nm was measured using a plate reader (Multiskan RC Version 6.0, Lab systems). The CAT activity in the samples was calculated from the standard curve and expressed in $U \cdot mL^{-1}$.

Analysis of malondialdehyde concentration

Malondialdehyde concentration, indicating the intensity of lipid peroxidation processes, was measured by the method of Rice-Evans et al. (1991) [29] as modified by Atmaca et al. (2004) [30]. To the analyzed serum and one of the controls containing 200 µL of distilled water, the following reagents were added: 20 µL of 2% BHT (butylhydroxytoluene) in ethanol, 1 mL of 15% TCA (trichloroacetic acid) in 0.25 M HCl, and 1 mL of 0.37% TBA (thiobarbituric acid) in 0.25 M HCl. In the second control sample TBA was replaced by 1 mL of distilled water. The samples were vortexed and heated in a water bath at 100°C for 10 min. After cooling, the samples were centrifuged. The absorbance in the supernatant was measured at 535 nm against distilled water as control. The obtained absorbances were corrected by subtracting the

absorbances of controls with TBA replaced by distilled water. Malondialdehyde concentration in the samples was calculated using the absorbance coefficient (156 mmol−1•cm−1). The concentration was expressed in μM.

Statistical analysis

Statistical analyses were performed with The R statistical package version 4.0.2. Regarding quantitative parameters, the results are displayed as minimum and maximum values, quartiles (Q1, Q3), medians, arithmetical averages and standard deviations. Data were analyzed for normal distribution. Those that did not exhibit normal distribution were analyzed with non-parametric tests (U-Mann-Whitney). Normally distributed data were compared with a Student-t test. Correlations were analyzed with Spearman rank correlation tests. The coefficient of significance was set at $\alpha < 0.05$ and statistical significance at $p < 0.05$ [31].

RESULTS

There were no statistically significant differences between the control and abnormal spermiogram groups in terms of antioxidant enzyme activity (SOD, CAT).

Malondialdehyde values and glutathione reductase activity measured in the serum of patients in the healthy group were higher than in the group with spermiogram disorders (Fig. 1).

DISCUSSION

Reactive oxygen species produces extensive protein damage, cytoskeletal modifications and inhibit cellular mechanisms. However, on the other hand ROS are fundamental mediators of physiological sperm function, such as signal transduction mechanisms that influence fertility. Reactive oxygen species can have positive effects on sperm and the concentration functions depending on the nature and the concentration of the ROS involved. They are necessary in regulating hyperactivation and the ability of the spermatozoa to undergo the acrosomal reaction [32].

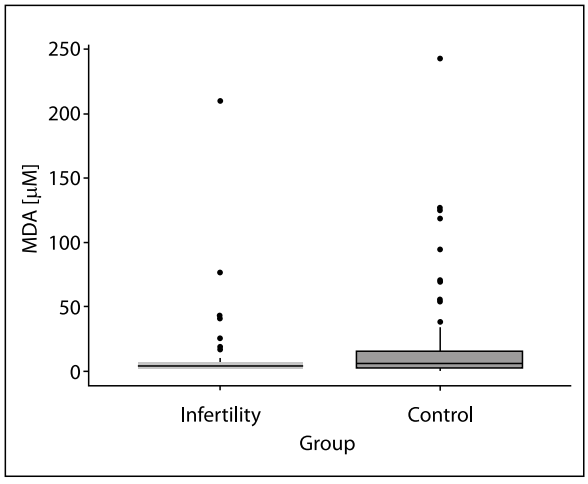

Figure 1. Malondialdehyde (MDA) concentration in the group of healthy and infertile patients

Impaired sperm activity, function and morphology can occur when levels of ROS or other free radicals are significantly elevated, while the body's antioxidant capacity is reduced [33, 34]. Elevated levels of ROS are found in 25–80% of infertile men, which is further associated with low levels of antioxidants in semen for men without fertility disorders [33, 35]. Reactive oxygen species causes a decrease in semen parameters by damaging sperm DNA or by initiating lipid peroxidation in membrane structures, which has a negative impact on sperm motility and the ability to fertilize oocytes [36]. Sources of ROS are many. These include activated leukocytes from inflammatory processes, immature spermatogenesis cells with abnormal morphology, coexistence of varicocele or cryptorchidism. In addition to factors independent of the patient, lifestyle, addictions, environmental and occupational exposures, and diet are also sources of excessive ROS [34].

Studies over the past two decades provide significant evidence to support the concept that excessive production of ROS is one of the causes of abnormal semen parameters and sperm damage [3, 11–14]. Male germ cells are extremely vulnerable to oxidative stress as the sperm membrane is rich in unsaturated fatty acids and lacks the capacity for DNA repair. Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA) and their cytoplasm contains low concentrations of the scavenging enzymes [27].

As spermatozoa has relatively low intracellular antioxidant activity, the enzymatic antioxidants present in seminal plasma, meaning SOD, CAT and GR, therefore play a very important role. In addition, several non-enzymatic antioxidants also contained in seminal plasma, such as ascorbic acid, uric acid and alpha-tocopherol, play a supporting function [37].

Sperm antioxidant defense is enhanced by MDA, which, as a breakdown product formed during lipoperoxidation, is one of the primary factors determining the intensity of this process by which it determines the severity of oxidative stress [38, 39]. The phenomenon of lipid lipoperoxidation disrupts the basic parameters of sperm, causing impairment of membrane integrity, motility and overall sperm metabolism [40].

Excess production of ROS can trigger the phenomenon of lipid peroxidation through non-enzymatic or enzymatic mechanisms. Lipoperoxidation products exhibit high biological activity and are capable of inducing cell death. Undoubtedly, the balance between ROS generation and elimination is a decisive factor. Therefore, under conditions

Table 1. Malondialdehyde (MDA) concentration and superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities in the infertility group (n = 159) and in the control group (n = 162)

Parameter	Control group							Infertility group							
	x	SD	Min	Q_1	Me	Q_3	Max	x	SD	Min	Q_1	Me	Q_3	Max	p value*
MDA [μm]	22.65	42.5	0.21	2.68	5.21	14.59	242	10.94	30.66	0.21	1.8	2.9	6.2	209	0.0136
CAT activity [nmol·min^{-1}·mL^{-1}]	58.98	91	2.46	23.51	40.4	69.22	1011.6	81.82	137.61	0.0	28.18	40.9	100.35	159.9	0.7248
SOD activity [U·mL^{-1}]	2.47	2.59	0.0	0.49	1.57	3.89	14.59	8.04	34.03	0.01	0.82	2.6	4.22	227.95	0.0508
GR activity [U·mL^{-1}]	26.09	17.6	0.0	14.43	20.94	33.11	89.14	28.48	24.74	1.7	13.41	19.7	38.2	114.61	0.0368

SD — standard deviation; Min — minimum value; Q — quartile; Me — medians; Max — maximum value; *Mann-Whitney U test

of limited lipoperoxidation, cell survival is promoted (the process itself stimulates the production of an "adaptive response", which manifests itself in the mobilization of antioxidant systems). In contrast, under toxic conditions (high levels of lipid peroxidation), the processes of apoptosis and cell necrosis are promoted [41].

Oxidative stress can be evaluated in different biological samples (plasma, serum, follicular/peritoneal/seminal fluid), obtaining an accurate picture of redox status. Blood and plasma redox status alterations have been reported in infertile men, as recently described in the study showing higher blood leukocytes ROS production, increased plasma lipid peroxidation (LPO) and reduced plasma total antioxidant capacity (TAC) in oligoasthenozoospermic men compared to healthy subjects [42]. In line with this, several findings also suggest that ROS-mediated sperm oxidation may induce cellular dysfunctions, affecting spermatozoa concentration, total number and motility [20, 43].

Although MDA levels may not be correlated with sperm DNA fragmentation and oxidation, suggesting that some fundamental parameters for sperm quality may remain independent of MDA [44], previous studies have emphasized the link between intense lipid peroxidation, elevated MDA levels, deteriorating sperm quality and overall reproductive potential [38, 39].

In these studies, it was found that higher levels of MDA in plasma and seminal fluid of infertile men correlated with semen parameters, thus indicating that redox status affects procreative capacity [38, 39, 45].

The data obtained in our study shows that serum MDA levels in men with normozoospermia were higher (22.65 μM) than in those with fertility disorders (10.94 μM, p = 0.013); (Tab. 1.). Our results are consistent with those of Kasperczyk et al. (2016) [46]. Morales et al. [47] note that elevated MDA levels can also be interpreted as a kind of adaptive mechanism. In some organisms, especially with undisturbed regulation of redox signaling, MDA can stimulate regulatory genes or even participate in cellular protection under oxidative stress. Therefore, transient increases in MDA may serve as a defensive signaling factor that participates in mobilizing antioxidant mechanisms to counter ROS. These suggestions led us to consider the temporary increases in MDA concentration as a protective mechanism rather than as an indicator of damage. This is an interpretation that may be valid for the healthy male controls analyzed in our study (Tab. 1). In addition, some key methodological aspects may determine the usefulness, or lack thereof, of the obtained results of MDA concentrations for specific analytical and research questions [47]. In the case of continuous exposure to oxidative stress, certain factors responsible for it tend to cause an increase in MDA concentrations in a dose-dependent manner. In such situations, the opposite effect can be observed with respect to the enzymatic activity of antioxidants, which can be significantly reduced [48].

In other published data, increased lipid peroxidation positively correlates with impaired spermatogenesis and reduced semen parameters and its marker MDA is elevated in infertile patients compared to patients without fertility disorders [49–51]. In other studies, MDA levels do not differ between fertile and infertile patients, moreover, it did not change under the influence of introducing additional antioxidant factors such as zinc supplementation [52]. With reference to the literature data, the result in terms of serum MDA levels in men with normozoospermia in the presented study does not seem controversial.

Shamsi et al. [53] found that blood levels of SOD and GSH positively correlated with sperm count and motility, while elevated MDA levels were associated with altered sperm morphology. Another study proves that plasma TAC significantly and positively correlated with both seminal fluid TAC and semen parameters [54], indicating that plasma redox status reflects the redox status of the seminal fluid microenvironment and sperm quality.

Our study proves that the assay of serum antioxidant enzyme activity (SOD, CAT), does not seem to be useful in the diagnosis of male infertility. There were no differences in the activity of these enzymes between the analyzed groups.

Although other authors have reported that the determination of antioxidant enzyme activity could be used to identify patients with oxidative stress and thus those who may be eligible for antioxidant treatment [55–57]. Further analyses and a comprehensive diagnostic and therapeutic consensus are needed.

CONCLUSIONS

It is known that oxidative stress is strongly associated with sperm dysfunction and represents a new pathological mechanism of male infertility [8, 20–22]. Numerous studies on this issue point to the need to develop new methods and diagnostic strategies for assessing male fertility. Along with assessing oxidative stress in seminal fluid, monitoring the redox status of blood could provide a new potential and less invasive practice for clinicians to assess sperm quality and fertilizing capacity. Although our study did not demonstrate the usefulness of the blood tests which were analyzed, further studies are needed to explore the potential use of assessing redox parameters to develop new diagnostic and therapeutic approaches for male infertility.

An interesting aspect of our study is the observed increase in GR in the group of patients with infertility, which requires further analysis, as we did not find studies on this issue in the available literature.

Article information and declarations

Data availability statement

The data presented in this study are available on request from the corresponding author.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Bioethical Commission of the Collegium Medicum of Nicolaus Copernicus University in Torun, Poland. Samples were collected under permit KB 674/2010; No. KB 427/2014 and No. KB 365/2015.

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Author contributions

M.S. and K.W. — conceptualization; M.S. — methodology; J.S. — software; R.S. and P.K. — validation; J.S. and M.A. — formal analysis; M.S., K.W., R.S., R.J., P.K., J.S., A.S. and M.A. — investigation; M.S. — resources; K.W. — data curation; M.S. and K.W. — writing, original draft preparation; M.S., K.W. and M.A. — writing, review and editing; M.A. — visualization; K.W. — supervision; M.A. — project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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