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**Correlation of oxidative stress markers and semen parameters
with the outcome of *in vitro* fertilization**

Корелација маркера оксидативног стреса и параметара спермограма
са исходом вантелесне оплодње

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Correlation of oxidative stress markers and semen parameters with the outcome of *in vitro* fertilization

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SUMMARY

Introduction/Objective The aim is assessing the effect of oxidative stress parameters on sperm count, motility, and morphology of spermatozoa, and the influence of different semen parameters on the outcome of *in vitro* fertilization (IVF) procedure - the number of fertilized oocytes, fertilization, and pregnancy rate of female partners.

Method In 52 male patients superoxide dismutase (SOD), malondialdehyde (MDA) and sulfhydryl (-SH) groups were determined in serum, before beginning the IVF cycle. Semen samples were collected after 2–3 days of sexual abstinence.

Results Patients were divided into two groups, the normozoospermic, and those with pathological sperm findings. The second group was divided into oligozoospermic, asthenozoospermic, and teratozoospermic groups. SOD activity was lower, while MDA and -SH groups, while not significantly, was higher in groups with sperm parameter disorders. Fertilization rate was significantly lower in the group of asthenozoospermia and teratozoospermia ($p = 0.034$), as well as delivery rate ($p = 0.020$). The group with oligozoospermia had significantly lower delivery rate ($p = 0.013$).

Conclusion Our study found higher fertilization and delivery rate in men with normozoospermia. However, no significant correlation between OS markers and semen parameters was found.

Keywords: *In vitro* fertilization, oxidative stress, sperm parameters

САЖЕТАК

Увод/циљ Циљ рада била је процена утицаја параметара оксидативног стреса на број, покретљивост и морфологију сперматозоида, као и утицај различитих параметара спермограма на исход поступка *in vitro* фертилизације (IVF)- број фертилисаних ооцита, стопе фертилизације и порођаја.

Метод Код 52 мушка партнера одређиване су вредности SOD, MDA и -SH група у серуму, пре започињања IVF поступка. Узорци семена сакупљани су након 2–3 дана абстиненције.

Резултати Пацијенти су били подељени у две групе: група са нормозооспермијом и група са патолошким налазом спермограма. Друга група је затим подељена на подгрупе: олигозооспермија, астенозооспермија и тератозооспермија. Активност SOD била је нижа, док су вредности MDA и -SH група биле више у групама са поремећеним параметрима спермограма, али не статистички значајно. Стопа фертилизације била је значајно нижа у групи са астенозооспермијом и тератозооспермијом ($p = 0.034$), а такође и стопа порођаја ($p = 0.020$). Група са олигозооспермијом имала је значајно нижу стопу порођаја ($p = 0.013$).

Закључак У овој студији пронађене су више стопе фертилизације и порођаја у групи са нормозооспермијом. Није било значајне корелације између параметара оксидативног стреса и спермограма

Кључне речи: *In vitro* фертилизација, оксидативни стрес, параметри спермограма

INTRODUCTION

Approximately 15 % of couples of reproductive age have problem with infertility and half of the cases are attributed to the male factor, while one of the mechanisms proposed for idiopathic male infertility is oxidative stress [1]. The diagnosis of male infertility is based on analysis of semen parameters: concentration, motility, and morphology of spermatozoa in the ejaculate.

Sperm cells are the first cells that are reported to be susceptible to oxidative damage. In the article printed in 1943, MacLeod confirmed the rapid loss of sperm motility when incubated in medium with increased concentration of oxygen [2]. Recent studies have also found an increase in reactive oxidative species (ROS) levels in 30–80 % of infertile men [3, 4]. ROS are natural products of cellular metabolism, that at physiological concentrations are

the essential requirements for the spermatozoa in the processes that lead to successful fertilization: ROS trigger sperm hyperactivation and capacitation [5]. Physiological oxidative conditions are necessary for the sperm maturation, binding to the zona pellucida, acrosomal reaction and subsequent fusion of sperm and oocytes [6, 7]. That indicates that ROS itself has no adverse effect, except when the levels are elevated. Available data on the impact of oxidative stress on sperm are largely based on measuring the levels of malondialdehyde (MDA) in semen. Since ROS have both physiological and pathological functions, the human body has developed a defense system to maintain its concentration in a certain range. Due to the size and small volume of the cytoplasm, as well as the low concentrations of the enzyme cleaners, sperm has limited antioxidant defense properties [8]. Antioxidant profile in the blood in relation to the antioxidant profile and quality of spermatozoa is less investigated. Correlation between superoxide dismutase (SOD) levels in the blood and sperm number, as well as glutathione levels and sperm progressive motility, suggests that these parameters may be valuable biochemical markers in assessing reproductive and functional capacity of sperm [8].

Most of studies examined ROS parameters in semen; there are only few studies that analyzed oxidative stress parameters in serum, or both in seminal plasma and serum. Serum oxidative stress markers as well as antioxidant profile showed correlation to sperm parameters, showing that serum can be a valuable tool in evaluation of oxidative stress in men. [8–11]

The aim of this study was to examine the association between oxidative stress parameters, MDA, SOD and -SH groups serum levels in male partner with semen parameters, as well as the influence of different sperm parameters and outcome of *in vitro* fertilization.

MATERIALS AND METHODS

Study subjects and sample collection

The prospective clinical study was conducted at the Clinic of Gynecology and Obstetrics, Clinical Center of Serbia. We recruited 52 male patients, admitted for fertility treatment. All investigated patients agreed to participate in the study and signed an informed consent for all the undertaken procedures. The study was approved by The Ethics Committee

of the Faculty of Medicine, University of Belgrade. Inclusion criteria were age 24–45 years, BMI 18–30 kg/m², no chronic illness, radiotherapy or chemotherapy. Patients with azoospermia were excluded from the study. Female partners were aged 18–40 years, BMI 18–30 kg/m², with regular menstrual cycles from 25–32 days, without any medical disease or endometriosis stage III and IV. Infertility cause was categorized as male and unknown. The protocols of stimulation were determined individually. After ovarian stimulation and oocyte retrieval, methods of insemination were in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or a combined method. In assessing the quality of embryos, the Istanbul consensus clinical embryologists' criteria were used as the reference frame [12]. Embryo transfer was performed transcervically on day two or three after the oocyte retrieval. Pregnancy rate were calculated per embryo transfer. Pregnancy was diagnosed by positive serum β -hCG (> 50 IU/ml) 14 days after the embryo transfer.

Semen was obtained by masturbation technique after 48–72 hours of sexual abstinence. Samples were collected into sterile containers for immediate transportation to the laboratory. All semen samples were evaluated in the same laboratory. After liquefaction at 37°C for 30 min, routine semen analysis (liquefaction time, volume, pH, viscosity, sperm count, motility, and morphology) was carried out after liquefaction, as per the WHO guidelines [13]. Samples were categorized on the basis of sperm count into normozoospermic and oligozoospermic, and on the basis of sperm motility into normozoospermic and asthenozoospermic. Similarly, on the basis of sperm morphology, they were categorized as normal and teratozoospermic for analysis purpose. Based on semen analysis we divided patients into two groups: those with normal semen analysis (normozoospermia) and those with pathological sperm finding. The second group was divided into three groups: oligozoospermic, asthenozoospermic and teratozoospermic group.

Blood samples for oxidative stress parameters were obtained from each patient prior to stimulation commencement in the female partner. After separation, the serum / plasma was frozen and stored at a temperature of -70°C. After incomplete defrosting, preparation of the serum for the analysis was carried out by homogenization and centrifugation. The following parameters of oxidative stress groups were determined as described in detail in our previous study [14]: concentrations of MDA, the activity of total SOD, and the concentration of total sulfhydryl (-SH).

Statistical analysis

For statistical analysis of the obtained data, the Statistical Package for the Social Sciences Version 22.0. (IBM Corp. Armonk, NY, USA) was used and differences were considered statistically significant at a probability level less than 0.05 for all tests. Results were presented as arithmetic mean and, \pm standard deviation for variables with normal distribution and as median and interquartile range for other variables. Categorical variables are presented as relative or absolute frequency. Testing of distribution was carried out by Kolmogorov–Smirnov analysis. Comparison of the mean values of independent groups of data was performed by Student's t test and ANOVA analysis with Tukey's post hoc test for the differences between subgroups. For parameters without normal distribution, a test of significance between groups was performed using the Mann–Whitney test or Kruskal–Wallis test with post hoc Mann–Whitney test. Comparison of two dependent populations was performed by the Wilcoxon signed - rank test for data without normal distribution. Analysis of categorical values was performed using the χ^2 test.

RESULTS

Oxidative stress parameters: MDA, SOD and -SH groups in males' serum and the outcome of IVF in female partner: number of fertilized oocytes, fertilization, and pregnancy rate are given in Table 1. In 52 men, we examined changes in the OS parameters and antioxidant protection depending on the disorder in sperm parameters and we compared them to normal semen analysis. No significant difference in the examined parameters between the groups was found, although the value of MDA were slightly lower in the group with normozoospermia compared to pathological findings (0.52 vs 0.57, $p = 0.254$), while the value of SOD (26.18 vs 24.12, $p = 0.348$) and -SH groups (0.46 vs 12:43, $p = 0.138$) were slightly higher in the group with normozoospermia (Table 2). As shown in Table 3, when we compared the individual findings of normozoospermia with findings of oligozoospermia, teratozoospermia, and asthenozoospermia, there was no significant difference. We also compared the number of fertilized oocytes, fertilization rate and outcome of pregnancies in female partners of examined male partners, depending on sperm parameters. A number of fertilized oocytes did not differ between groups. The fertilization rate was higher in male partners with normozoospermia, compared to abnormal semen analysis (68 % vs 50 %, $p =$

0.102) (Table 4), as well as the pregnancy rate (44.1 % vs 40 %, $p = 0.756$). In the group with normozoospermia, female partners who conceived had delivered a healthy child in 83 %, comparing with 62.5 % in female partners of men with all other sperm parameters, which was significantly lower ($p = 0,034$). When we separated group with disorder in sperm parameters, there was no significant difference concerning the number of fertilized oocyte ($p = 0.864$), as well as fertilization rate ($p = 0.475$) between normozoospermia and oligozoospermia. In female partners of men with asthenozoospermia and teratozoospermia there was a significantly lower fertilization rate comparing to normozoospermia group ($p = 0.034$) (Table 5). The group with oligozoospermia had delivery rate significantly lower ($p = 0.013$).

DISCUSSION

In our study, we did not find significant difference in the examined changes in oxidative stress parameters and antioxidant protection between men with normal semen analysis and those with the disorder in sperm parameters. However, the value of MDA was higher in the group with pathological sperm finding, while the value of SOD and -SH groups were higher in the group with normozoospermia. When we compared oligozoospermia, teratozoospermia, and asthenozoospermia to normozoospermia group, results were similar. These trends in investigated parameters values are in accordance with some other studies [8, 9, 15]. Huang et al. [15] found higher concentrations of MDA in men with asthenozoospermia and oligoasthenozoospermia compared to normozoospermic men, showing similar finding as in our study. Low levels of MDA in the seminal plasma were associated with an increased progressive motility of sperm and a positive correlation between elevated levels of MDA and abnormal morphology of sperm were also found, which is consistent with the findings of other authors [16]. Different impairments of sperm cells as well as male infertility can be caused by increased lipid peroxidation. MDA is an indicator of the lipid peroxidation and may be a diagnostic tool in infertility, as well as a predictor of ART procedures success [17].

In results of studies that are comparable to our, correlation between the level of SOD in the serum and the sperm number was found in patients with pathological findings [8]. This group of investigators also found a significant correlation between the level of GSH in the serum and sperm progressive motility between patients with infertility and normal controls In

the study of Benedetti et al. [9] that estimated antioxidants profile in the plasma of fertile and infertile patients, lower antioxidants were found in infertile males, positively correlated with the concentration, motility, and morphology of spermatozoa. In the paper of Mahanta et al. [18] the level of lipid peroxides in the blood of the infertile group was significantly higher compared to the fertile one, while the activity of SOD and GPX in the blood was significantly lower compared to fertile men. Some studies have not observed the difference in GSH between fertile and infertile males [19], while other have observed significantly reduced GSH levels in the seminal plasma of the infertile men as compared to fertile ones [20, 21].

Although the analysis of sperm is routine, we do not get information about the functional capacity of sperm, consequently, no semen parameter by itself can predict the possibility of success of ART procedures. However, the percentage of sperm with normal morphology is in positive correlation with fertilization and pregnancy rates in IVF [22, 23]. We observed significantly lower fertilization rate in patients with asthenozoospermia comparing to normal finding. Fertilization rates significantly vary in the findings of normospermia compared to pathological semen findings and some studies found no association [24]. In patients with severe teratospermia [25], oligo - or azoospermia [27], the DNA fragmentation and the degree of aneuploidies of sperm were significantly higher, as well as a higher percentage of aneuploidy in embryos was found [27]. Even in these findings, fertilization can occur, but the rate of miscarriages is significantly higher [28]. However, there is a wide variation among samples from one individual [29], and therefore more sophisticated tests of sperm function or selection are needed for improving the outcome of IVF procedures.

The present study has few limitations; it would be more relevant when we have a larger number of patients. Also, we are planning to include information about lifestyle habits, such as eating habits, alcohol intake, smoking, use of supplements etc. in the future studies. Beside assessment of oxidative stress status from serum, data from sperm would be beneficial too.

CONCLUSION

In summary, our result suggests that abnormal semen parameters affect the outcome of IVF. Fertilization rate was lower in the group with asthenozoospermia, while the delivery rate

is lower in oligozoospermia, asthenozoospermia, and teratozoospermia. However, no significant correlation between OS markers and semen parameters was found.

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Conflict of interest: None declared.

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Table 1. Oxidative stress parameters in serum of male partners and in vitro fertilization outcome in female partners

Parameters	Values
SOD (U/L)	25.17 (22.4–28.82)
MDA ($\mu\text{mol/L}$)	0.56 (0.48–0.65)
-SH groups (mmol/L)	0.44 (0.39–0.52)
Number of fertilized oocytes (n)	2.5 (1–6)
Fertilization (%)	54.5 (33.3–81.8)
Pregnancy rate (%)	41

SOD – superoxide dismutase; MDA – malondialdehyde; -SH – sulfhydryl groups.

The median (25th and 75th percentile) are shown. The statistical analysis was made using Mann–Whitney test.

Table 2. Oxidative stress parameters in serum of men with normozoospermia and pathological sperm finding

Parameters	Normozoospermia N = 30	Pathological finding N = 20	p
SOD	26.18 (22.63–28.89)	24.12 (22.37–29.08)	0.348
MDA	0.52 (0.47–0.64)	0.57 (0.51–0.65)	0.254
-SH groups	0.46 (0.41–0.53)	0.43 (0.38–0.47)	0.138

SOD – superoxide dismutase; MDA – malondialdehyde; -SH – sulfhydryl groups.

The median (25th and 75th percentile) are shown. The statistical analysis was made using Mann–Whitney test.

Table 3. Oxidative stress parameters in serum of male partners with normozoospermia and oligozoospermia, asthenozoospermia and teratozoospermia

Parameters	Normozoospermic N = 30	Oligozoospermic N = 17	Asthenozoospermic N = 16	Teratozoospermic N = 12
SOD	26.18 (22.63– 28.89)	24.12 (22.36–29.08)	23.9 (22.4–29.3)	26.0 (23.43–29.65)
MDA	0.52 (0.47–0.64)	0.57 (0.51–0.65)	0.58 (0.52–0.65)	0.58 (0.51–0.64)
-SH groups	0.46 (0.41–0.53)	0.43 (0.38–0.47)	0.41 (0.34–0.46)	1.38 (1.28–1.46)

SOD – superoxide dismutase; MDA – malondialdehyde; -SH – sulfhydryl groups.

The median (25th and 75th percentile) are shown. The statistical comparison was made using Mann–Whitney test.

Table 4. Number of fertilized oocytes and fertilization rate in female partners of men with normozoospermia and all others sperm parameters

Parameters	Normozoospermia N = 30	Pathological finding N = 20	p
Fertilized oocytes (n)	2.5 (1.8–5.6)	2.5 (1–6)	0.955
Fertilization (%)	68	50	0.102

The median (25th and 75th percentil) are shown. The comparison was made using Mann–Whitney test

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Table 5. Number of fertilized oocytes and fertilization rate in female partners of men with normozoospermia and oligozoospermia, asthenozoospermia and teratozoospermia

Parameters	Normozoospermic N = 30	Oligozoospermic N = 17	Asthenozoospermic N = 16	Teratozoospermic N = 12
Fertilized Oocytes	2.5 (1.8–5.6)	2.5 (1–6)	2.5 (1–5.8)	2 (1–3)
Fertilization %	68 (39.1–100)	50 (25.2–74.6)	35.7 (25–68.6)	50 (25–75)

The median (25th and 75th percentile) are shown. The comparison was made using Mann–Whitney test