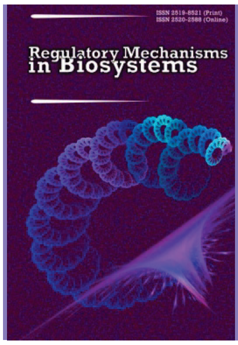

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Indicators of oxidative-nitrosative stress in men with idiopathic infertility and infertility associated with rheumatoid arthritis

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Infertility is a worldwide problem affecting 15% of couples. About 30% cases of male infertility are considered idiopathic associated with factors not limited to testicular dysfunction. Male infertility and rheumatoid arthritis are often associated and patients with rheumatoid arthritis have a lower fertility rate. The exact relationship between rheumatoid arthritis and male infertility has yet to be revealed. The aim of the study is to assess the MDA level and the activity of NO-synthase isoforms and arginase in men with idiopathic infertility and infertility associated with rheumatoid arthritis. Patients were divided into 2 groups: group 1 – 73 patients with idiopathic infertility; group 2 – 68 infertile men with a systemic autoimmune disease (rheumatoid arthritis). The MDA level in blood plasma of patients with idiopathic infertility and infertile men with rheumatoid arthritis was significantly higher than in the control group (fertile men). The cNOS activity in seminal plasma and blood lymphocytes decreased in men with idiopathic infertility and infertility associated with rheumatoid arthritis compared to the control group. The decrease in cNOS activity was accompanied by drastic increase in iNOS activity. The iNOS/cNOS ratio increased in men with idiopathic infertility and men with infertility associated with rheumatoid arthritis. Similar, but more drastic changes in iNOS/cNOS ratio were found in blood lymphocytes. The arginase activity in seminal plasma was decreased in patients with idiopathic infertility compared to the healthy controls. However enzyme activity in men with infertility associated with rheumatoid arthritis had a tendency to increase, but these changes are not significant. Similar changes were observed in blood lymphocytes. It was found that idiopathic infertility is associated with a decrease in the activity of non-oxidative, arginase metabolism and the dominance of NO-synthase, which leads to the hyperproduction of nitric oxide. In men with infertility associated with rheumatoid arthritis an increase in the arginase/NOS ratio in lymphocytes is noted, which is largely explained by the sharp activation of arginase activity in blood lymphocytes. The arginase/NOS balance and iNOS/cNOS ratio may can be used as indicators of oxidative-nitrosative stress in men with idiopathic infertility and infertility associated with rheumatoid arthritis.

Keywords: male infertility; idiopathic infertility; rheumatoid arthritis; oxidative stress; nitrosative stress; NO-synthase; arginase.

Introduction

Infertility is a worldwide medical, demographic and social problem affecting 15% of couples. Male factors account for almost 50% of all cases (Wagner et al., 2018). Male infertility diagnosis is based on standard semen parameters analysis according to the WHO guidelines. Despite advances in diagnosis and medical treatment male infertility over the past few decades, about 30% cases of male infertility are still considered idiopathic (Agarwal et al., 2019). The reasons of idiopathic infertility can occur due to the summation of negative factors, often unknown today and related not only to fertility (Tahmasbpour et al., 2014). Impaired spermatogenesis is often associated with factors not limited to testicular dysfunction (Puzuka et al., 2021).

Male infertility and rheumatoid arthritis are often associated and patients with rheumatoid arthritis have a lower fertility rate. Rheumatoid arthritis (RA) is an autoimmune disease characterized by an immune disorder regulation with characteristic signs of chronic inflammation and joint pain (Gibofsky, 2014). The exact relationship between RA and male infertility has yet to be revealed. However RA is believed to reduce a couple's potential for success in childbirth (Fattah et al., 2020). The male gonad may be affected by the disease activity and antisperm antibody (Tiseo et al., 2016). Moreover, it is still unclear whether RA itself or related therapeutic drugs can affect fertility. There is probably a cross-link between the disease on RA and fertility (Provost et al., 2014). Thus, now there is no clear idea how much RA causes infertility, is it also related to the influence of other factors, in particular, drugs used for treatment this pathology. An imbalance between the formation of reactive oxygen species (ROS) and activity of antioxidant protection systems may be one of the factors male infertility caused by sperm dysfunction (Mannucci et al., 2022). Under physiological conditions ROS are continuously produced and their concentrations are strictly regulated by means of enzymatic and non-enzymatic antioxidants (Chen et al., 2013). Hyperproduction of ROS is associated with a variety of male fertility complications, including idiopathic infertility (Du Plessis et al., 2015). According to literature data, ROS may be a contributing factor in 30-80% of male infertility associated with sperm damage which leads to a decrease in the fertilizing potential of spermatozoa (Bisht et al., 2017).

In addition to the important role of ROS in maintaining cellular homeostasis, an important regulatory role belongs to nitric oxide (NO) as a secondary messenger. It is a universal regulator of almost all physiological functions of the body. NO is considered a key factor in male fertility. However, the synthesis of NO in a high concentration can lead to the development of nitrosative stress. The synthesis of the NO from L-arginine is mediated by the few isoforms of NO synthase: endothelial, neuronal, and inducible, which differ in their location, nature of induction and are encoded by different genes. Neuronal (nNOS) and endothelial (eNOS) isoforms of enzyme are Ca^{2+}-dependent constitutive enzymes (cNOS). They are responsible for production of a physiological or basal small amount of NO. Inducible NOS (iNOS) is Ca^{2+}-independent and is not active under physiological condition. Expression of iNOS is induced by pro-inflammatory factors (Wu et al., 2022). The exact physiological and pathological role of NOS in male reproductive functions are based on its isoforms (Zhang et al., 2023). There is competitive to the NO-synthase pathway of L-arginine transformation with the participation of arginase (Bratt et al., 2011).

The aim of the study is to assess the MDA level and the activity of NO-synthase isoforms and arginase in men with idiopathic infertility and infertility associated with rheumatoid arthritis.

Materials and methods

The research was carried out at the urology department of the Lviv Regional Clinical Hospital (Lviv, Ukraine). Patients were divided into 2 groups: 73 patients with idiopathic infertility (group 1) and 68 infertile men with a rheumatoid arthritis (group 2). A detailed medical history of patients was obtained for all the men. The idiopathic infertility was diagnosed as the lack of fertilization during a year of the couple's life with no cause of the disease. Group 2 consists of patients with infertility (asthenozoospermia or leukocytospermia) and rheumatoid arthritis without other inflammatory diseases, oncological pathology or concomitant inflammatory diseases of the connective tissue.

Exclusion indications were azoospermia, hematospermia, necrozoospermia, testicular varicocele, genital infection, hormonal abnormalities, genetic diseases, diabetes or infertility over 10 years. Patients with any chronic disease and who reported alcohol abuse, smoking habits, drug consumption were excluded from the study. The control group consisted of 56 age-matched males with normo-zoospermia (normal semen parameters) according to the WHO criteria and confirmed parenthood. They were randomly picked. Before turning to the study, all patients gave informed consent to participate in the research. Terms of sample selection meet the requirements of the principles of the Helsinki Declaration on protection of human rights, Convention of Europe Council on human rights and biomedicine and the provisions of laws of Ukraine. Approval for the study was taken from the ethics committee of Danylo Halytsky Lviv National Medical University (protocol No 7 from 26 June 2023).

Semen samples from patients and control group were collected via masturbation after 3–4 days of abstinence and processed immediately upon liquefaction. All semen samples from patients and controls were collected into sterile containers. Semen analyses were performed according to World Health Organization criteria (2010). Blood samples was collected by venipuncture using sterile blood collection tubes from the elbow vein and stabilized with heparin (final dilution 1:100). Samples were collected in the morning, under conditions of physiological rest, on an empty stomach. Seminal fluid and blood plasma were stored in laboratory conditions at temperature of –20 °C he beginning of biochemical analysis, mostly for two weeks.

Determination of the concentration of malonic dialdehyde (MDA) was performed on the basis of its interaction with 2-thiobarbituric acid with the formation of chromogen with a maximum absorption in the red spectrum at a wavelength of 532 nm. NOS activity was determined by measuring the amount of NADPH oxidation (Onufrovych et al., 2024). For determination of Ca^{2+}-independent iNOS activity, 2 µmol of EGTA was added to the incubation mixture instead of $CaCl_2$. cNOS activity was calculated by subtracting iNOS activity from total NOS activity. Arginase activity was determined by measuring levels of urea production in a reaction mixture with the following composition (mmol/mL): L-arginine – 100, $MnCl_2$ – 2, Tris-HCl – 20 (pH 9.5). The reaction was stopped by adding 1 mL 50% trichloro-acetic acid. The urea concentration was determined after centrifugation in the supernatant by means of spectrophotometer at 520 nm according to the assay kit "Simko Ltd". Protein concentration in samples was determined by the Lowry method. MDA level and enzyme activities were measured by spectrophotometer V-1150 (China, 2023).

The results are presented as the mean ± standard error (x ± SE). Analysis of variance (ANOVA) was used to compare the difference in biochemical parameters between groups. Differences were considered statistically significant at $P < 0.05$.

Results

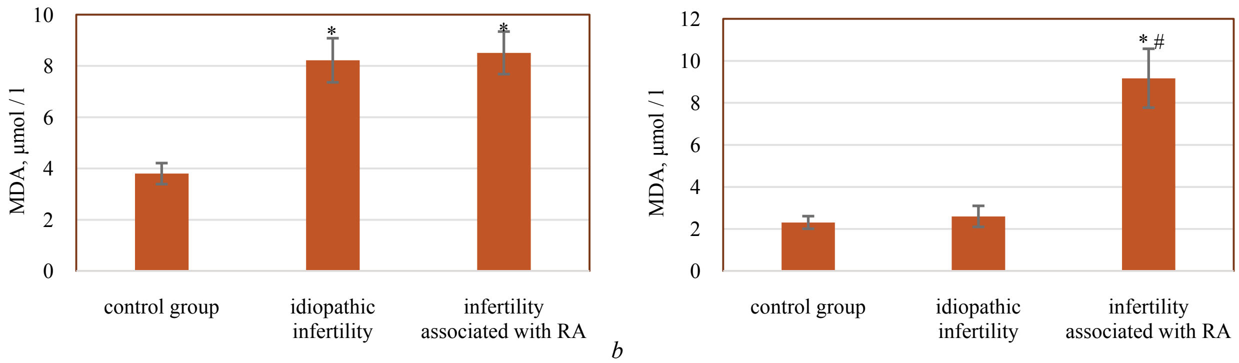
The state of indicators of oxidative stress in the blood serum and seminal plasma of infertile men was evaluated for content of malondialdehyde (MDA). It is a secondary product of polyunsaturated oxidation fatty acids, which is formed as a result of their degradation by ROS. The MDA level in blood plasma of patients with idiopathic infertility and infertile men with rheumatoid arthritis was significantly higher than in the control group (fertile men, Fig. 1).

The MDA level in these groups was 2.16–2.24-fold ($P < 0.001$) greater than in the control group. However, changes in MDA level in seminal plasma were significant only in patients with infertility associated with rheumatoid arthritis. The MDA level was 3.97-fold ($P < 0.001$) greater compared to the control group and 3.97-fold ($P < 0.001$) greater compared to the idiopathic infertility subjects.

The cNOS activity in seminal plasma was decreased by 2.82-fold ($P < 0.01$) in patients with idiopathic infertility and 1.57-fold ($P < 0.05$) in infertile men with rheumatoid arthritis compared to control group (Fig. 2). Similar changes in cNOS activity were found in blood lymphocytes, however a decrease in enzyme activity is less expressed.

The decrease in cNOS activity in men with idiopathic infertility and infertility associated with rheumatoid arthritis was accompanied by a drastic increase in iNOS activity (Fig. 3). The iNOS activity in seminal plasma increased by 21.88-fold ($P < 0.001$) and 23.66-fold ($P < 0.001$), respectively, in idiopathic infertility subjects and infertile men with rheumatoid arthritis as compared to the healthy controls. Similar but more expressed changes were observed in blood lymphocytes.

Next we evaluated the non-oxidative metabolism of L-arginine by arginase (Fig. 4). The arginase activity in seminal plasma was decreased by 1.60-fold ($P < 0.01$) in patients with idiopathic infertility compared to the healthy controls. However enzyme activity in men with infertility associated with rheumatoid arthritis had a tendency to increase, but these changes are not significant. Similar changes were observed in blood lymphocytes. The arginase activity in blood lymphocytes was decreased by 1.40-fold ($P < 0.05$) in patients with idiopathic infertility compared to the healthy controls. Infertile men with rheumatoid arthritis exhibited an increase in arginase activity (up to 2.78-fold, $P < 0.001$) when compared to the healthy controls.



a *b*

Fig. 1. The MDA level in blood plasma (*a*) and seminal plasma (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis

($x \pm SE$, N = 197): * – $P < 0.001$ changes are statistically significant compared to the control group (fertile men); # – $P < 0.001$ changes are statistically significant compared to idiopathic infertility subjects

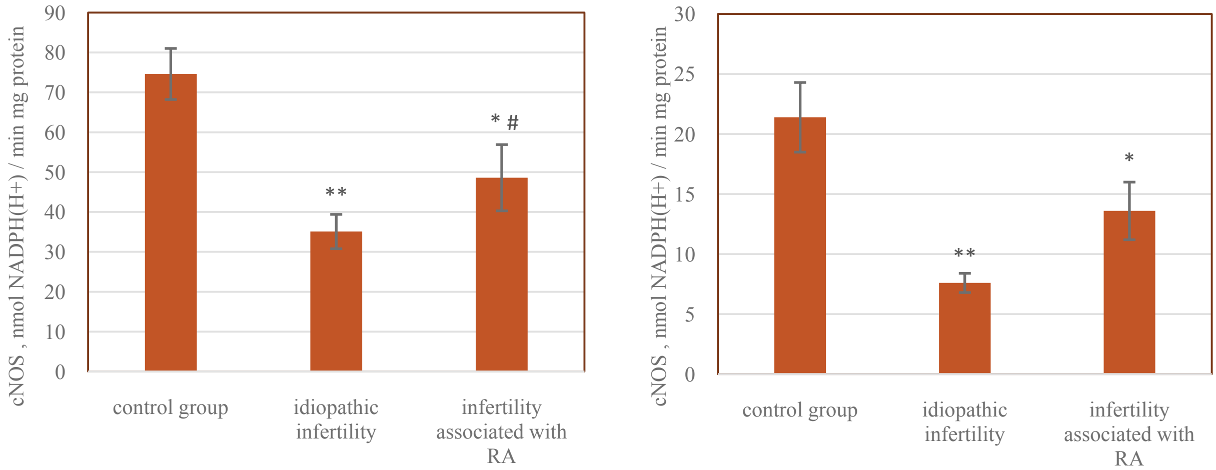


Fig. 2. The cNOS activity in seminal plasma (*a*) and blood lymphocytes (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis ($x \pm SE$, N = 197): * – $P < 0.05$; ** – $P < 0.01$ changes are statistically significant compared to the control group (fertile men); # – $P < 0.001$ changes are statistically significant compared to idiopathic infertility subjects

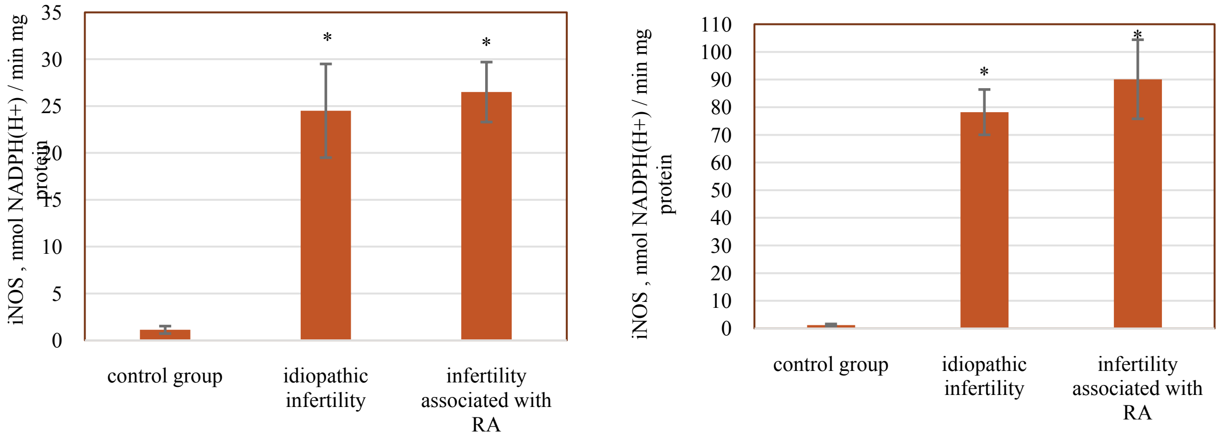


Fig. 3. The iNOS activity in seminal plasma (*a*) and blood lymphocytes (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis ($x \pm SE$, N = 197): * – $P < 0.001$ changes are statistically significant compared to control group (fertile men)

It should be noted that Ca^{2+}-dependent cNOS is expressed constantly. Instead, iNOS is not expressed in physiological conditions, it is activated only in inflammatory processes. Therefore, we studied the iNOS/cNOS ratio in patients of both groups (Fig. 5).

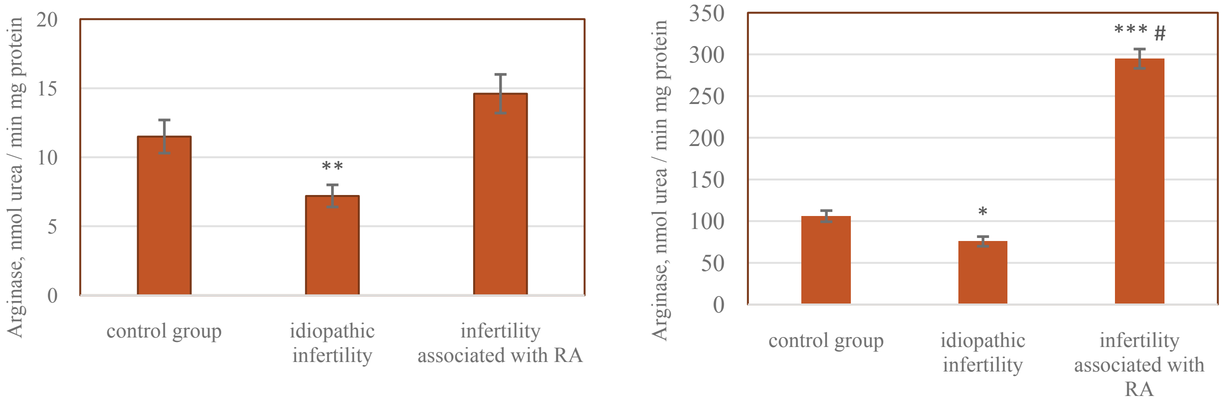
The iNOS/cNOS ratio increased by 61.60-fold ($P < 0.001$) in men with idiopathic infertility and by 37.23-fold ($P < 0.001$) in men with infertility associated with rheumatoid arthritis. Similar, but more drastic changes in iNOS/cNOS ratio were found in blood lym-phocytes.

Finally, we studied the ratio of arginase activity to the total NOS activity (Fig. 6). Men with idiopathic infertility exhibited a decrease in arginase/NOS ratio by 2.28-fold ($P < 0.05$) in seminal plasma and by 2.09-fold ($P < 0.05$) in blood lymphocytes when compared to the healthy controls. Contrary to this, the arginase/NOS ratio was 1.52-fold ($P < 0.05$) greater in blood lymphocytes of men with infertility associated with rheumatoid arthritis.

Discussion

At present, the number of infertile couples is increasing. It is assumed that in 80% of cases, idiopathic male infertility is caused by oxidative stress which develops as a result of an imbalance between ROS production and antioxidant capacity of semen. Sperm cells are extremely sensitive to oxidative stress, which leads to violation of the integrity of membrane due to high content of polyunsaturated fatty acids, its permeability. It was suggested that oxidative status of blood and seminal plasma are a valuable tool to improve the evaluation of sperm cells, their reproductive capacity in infertile men (Benedetti et al., 2012; Aitken et al., 2016). Therefore in the present study we examined indicators of oxidative-nitrosative stress in men with idiopathic infertility and infertility associated with rheumatoid arthritis in blood plasma (or blood lymphocytes) and seminal plasma.

It is now beyond doubt that the infertility is associated with development of oxidative and nitrasive stress and violation of the mechanisms of antioxidant protection (Nguyen-Powanda & Robaire, 2020; Evans et al., 2021; Aitken et al., 2022). MDA, which is a marker of oxidative stress, may be a diagnostic indicator for the prognosis of infertility. It is formed as a result of metabolism of arachidonic and other polyunsaturated fatty acids. MDA level represents intensity of lipid peroxidation induced by oxidative stress (Bergsma et al., 2022). In our previous study we revealed that MDA content was highest in patients with rheumatoid arthritis, which is probably due to leukocytospermia. We found a direct correlation between the MDA content and the concentration of leukocytes in seminal fluid ($r = 0.84$; $P < 0.05$) (Melnyk et al., 2022). MDA level in seminal fluid was negatively correlated with the viability and motility of sperm cells and positively correlated with acrosome abnormalities (Ben Abdallah et al., 2009; Benedetti et al., 2012). This confirms that oxidative damage to lipids of membranes impairs sperm quality (Benedetti et al., 2012). No significant correlation was found between MDA levels in serum and seminal plasma (Bergsma et al., 2022). In the present study an increased MDA level in seminal plasma was found only in patients with rheumatoid arthritis. There was no differences between MDA level in seminal plasma in men with idiopathic infertility and fertile men. This indicates other immune mechanisms involved in the pathogenesis of idiopathic infertility than oxidative stress.



a *b*

Fig. 4. The arginase activity in seminal plasma (*a*) and blood lymphocytes (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis ($x \pm SE$, N = 197): * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ changes are statistically significant compared to control group (fertile men); # – $P < 0.001$ changes are statistically significant compared to idiopathic infertility subjects

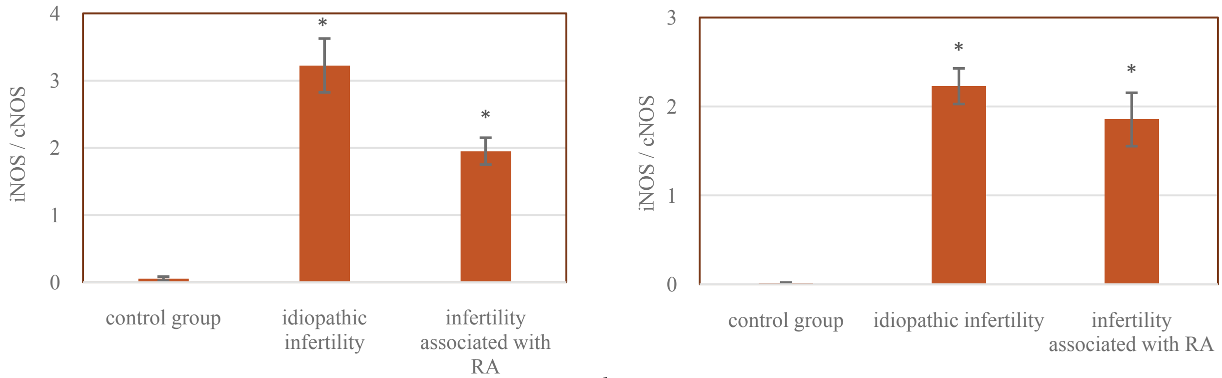


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Fig. 5. The iNOS/cNOS ratio in seminal plasma (*a*) and blood lymphocytes (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis ($x \pm SE$, N = 197): *– $P < 0.001$ changes are statistically significant compared to control group (fertile men)

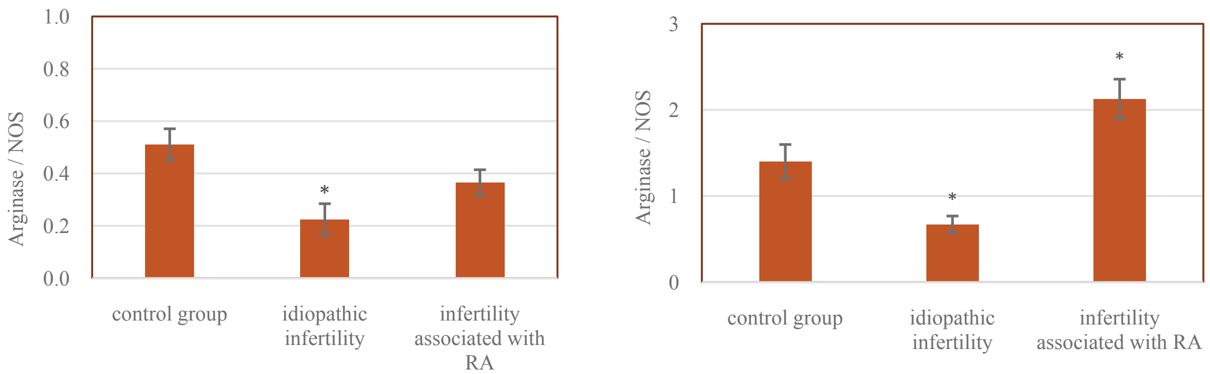

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Fig. 6. The arginase/NOS ratio in seminal plasma (*a*) and blood lymphocytes (*b*) of men with idiopathic infertility and infertility associated with rheumatoid arthritis ($x \pm SE$, N = 197): *– $P < 0.05$ changes are statistically significant compared to control group (fertile men)

It is known that human semen contains different enzyme and non-enzyme antioxidant systems that protect sperm cells from oxidative damage (Kowalczyk, 2022). In our previous studies we have demonstrated that activation of lipid peroxidation (evaluated as increased MDA level) was accompanied by a decrease in the glutathione peroxidase and glutathione reductase activity in infertile men with idiopathic infertility and with rheumatoid arthritis (Fafula et al., 2023).

NO plays an important role for the male reproductive system, including erectile functions, androgen secretions and sperm quality and functions. It is regarded as a key factor in male fertility (Dutta & Sengupta, 2022). NO plays a major role for the male physiological system as well as for sperm motility, maturity, quality and ability, and oocyte binding to sperm in physiological waterfalls such as erectile functions and androgen secretions. NO-synthases are the enzymes which ensure the production of nitric oxide (NO) from amino acid L-arginine. It should be noted that is Ca^{2+}-dependent cNOS is constantly expressed. Ca^{2+} independent iNOS is usually not expressed under physiological conditions. An increase in iNOS activity leads to overproduction of NO, which is the main precursor of reactive nitrogen species. Like ROS, they disrupt the cellular redox balance, may cause damage to susceptible biomolecules via peroxidative and nitration processes (Pérez-Torres et al., 2020; Dutta & Sengupta, 2022). A decrease in cNOS activity in seminal plasma in idiopathic infertility may be caused by a decrease in the expression of the gene or dysfunction of the enzyme under patho-logical conditions, violation of the ratio of substrates and/or cofactors necessary for the functioning of cNOS.

Since L-arginine is the substrate for NO-synthase, we evaluated the non-oxidative metabolism of this amino acid by arginase. A decrease in arginase activity in idiopathic infertility subjects, which could cause limitation of NO hyperproduction by competition for a common substrate with NOS, creates more favorable conditions for the functioning of iNOS. The obtained data indicate a decrease in the activity of non-oxidative arginase metabolism in idiopathic infertility subjects, which competes with oxidative NO-synthase metabolism of L-arginine. Similar findings were observed in sperm cells of men with different forms of pathospermia (Fafula et al., 2018). However, in men with infertility associated with rheumatoid arthritis there was sharp activation of arginase activity in blood lymphocytes.

The results of the present study should be considered in the context of its limitations. Patients of both studied groups represent a highly heterogeneous population. The present study investigated the MDA level and the activity of NO-synthase isoforms and arginase only with 197 cases. The possible role of other factors (for example lifestyle factors, infertility history etc.) have to be taken into account.

In general, the results we obtained indicate a violation of arginase/NOS balance, which leads to imbalance of regulatory function of NO. Increase in iNOS activity indicates hyperproduction of NO which has a pronounced cytotoxic effect as a result of formation of peroxynitrite. It is the product of the interaction NO and the superoxide anion radical and is capable of the destruction of almost all cell components. On the other hand, an increase in the iNOS activity and the development of oxidative and nitrosative stress is a consequence of the generation of ROS and reactive nitrogen species (Yousefniapasha et al., 2015). The present study proves the importance of NOS and arginase in the etiology and pathophysiology of idiopathic infertility and infertility associated with rheumatoid arthritis. It follows that inhibition of iNOS can be a potential therapeutic target in the treatment of infertility.

Conclusion

It was shown that MDA level and iNOS activity were significantly higher in seminal plasma of men with idiopathic infertility and infertility associated with rheumatoid arthritis. Increase in MDA level in blood lymphocytes was demonstrated only in men with infertility associated with rheumatoid arthritis. The iNOS/cNOS ratio was shifted toward iNOS activity in both studied groups. It was found that idiopathic infertility is associated with a decrease in the activity of the non-oxidative, arginase metabolism and the dominance of NO-synthase, which leads to the hyperproduction of nitric oxide. In men with infertility associated with rheumatoid arthritis an increase in the arginase/NOS ratio in lymphocytes is noted, which is largely explained by the sharp activation of arginase activity in blood lymphocytes. The arginase/NOS balance and iNOS/cNOS ratio may can be used as indicators of oxidative-nitrosative stress in men with idiopathic infertility and infertility associated with rheumatoid arthritis.

No potential conflicts of interest relevant to this article were reported.

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