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Original Scientific Paper

Role of antioxidants in the diminution of oxidative stress and amelioration of semen parameters in idiopathic male infertility

Vegim Zhaku[1], Sheqibe Beadini[2], Nexhbedin Beadini[3], Vjosa Xhaferi[1]

[1] Department of Physiology, Faculty of Medical Sciences, University of Tetova, Republic of North Macedonia*
[2] Department of Biochemistry, Faculty of Medical Sciences, University of Tetova, Republic of North Macedonia
[3] Department of Cell Biology and Human Genetics, Faculty of Medical Sciences, University of Tetova, Republic of North Macedonia
[*]Corresponding Author: vegim.zhaku@unite.edu.mk

Abstract

Antioxidants improve semen parameters in terms of concentration and motility, thus we decided to study their efficacy in men with idiopathic infertility. We enrolled 37 men undergoing idiopathic male infertility. The first semen sample was collected at the time of enrollment in the study. From this 0.5 ml of the sample was for standard semen analysis, 1.2 ml of the sample was for the separation of seminal plasma to evaluate oxidative stress (OS) parameters such as malondialdehyde (MDA) and protein carbonyl (PC). A second sample was collected after 6 months of antioxidant treatment. Mean, standard deviation, the Pearson correlation, and paired student t-test were used for statistical analyses. After six months of treatment, the mean sperm concentration before treatment was 13.84, after treatment 20.38, mean ±SD =-6.54 ±5.35. The mean of sperm motility before treatment was 27.16, after treatment 34.05, mean ± SD = -6.89 ± 11.72. Concentration and motility were significantly increased after 6 months of treatment ($p<0.001$). The mean level of MDA before treatment was 5.172, mean after treatment 3.968, mean ± SD = 1.204 ± 2.26. This shows significantly lower values after six months of therapy with antioxidants ($p<0.001$). Another oxidative marker denoted as PC was also lower after the treatment, but was not statistically significant ($p=0.0554$). We suggest that Antioxidants should be used on a routine basis in cases of idiopathic male infertility.

.*Keywords:* Male Infertility, Oxidative stress, Antioxidants, Semen parameters.

1. Introduction

It is estimated that 60% of married couples, having regular unprotected intercourse, achieve pregnancy after 6 months of co-habitation, 90% achieve pregnancy after 12 months, and 95% between 18 to 24 months (Agboola, 2001). The incidence of infertility is approximately 15% (Agarwal, Mulgund, Hamada, & Chyatte, 2015). Out of this percentage males and females contribute equally in 50% of cases respectively (Snarlip, Jarop and Belker, 2002). Normal reproductive function is essential for producing offspring and sexual satisfaction in males during their reproductive age (Zhaku, Beadini, Beadini and Murtezani, 2019). The American Society for Reproductive Medicine (ASRM) considers infertility as a disease which by definition is, "Any deviation from, or interruption of, the normal structure or function of any part, organ, or system of the body as manifested by characteristic symptoms and signs; the etiology, pathology, and prognosis may be known or unknown" (WAN, 2007). Several multiple causes threaten the physiology of the reproductive system including: varicocele, obstruction, ejaculatory failure, testicular failure, endocrinal, radiation, drugs, tobacco and alcohol use, environmental, sexual dysfunction, infection, genetic and cancer, but the biggest cause remains idiopathic at 32.6% (Naz and Mehnaz, 2017; Nieschlag, 2000; Harlev, Agarwal, Gunes, Shetty and du Plessis, 2015; Pizent, Blanka and Tanja, 2012; Jensen, Bonde and Joffe, 2006; Kolesnikova, Kolesnikov, Kurashova and Bairova, 2015). When we say idiopathic we mean oxidative stress (OS). This is an imbalance between pro-oxidants and antioxidants (Al-Gubory, Fowler and Garrel, 2010). It has been

proven in a lot of studies that high levels of reactive oxygen species (ROS) are correlated with unexplained male infertility. Also increasing levels of ROS cause chemical and structural modifications to sperm DNA, to protein, lipids in mitochondria and plasma (Shen, Chia and Ong, 1999; Agarwal, Makker and Sharma, 2008). Under physiological conditions spermatozoa produce small amounts of ROS, which are needed for capacitation, maturation of spermatozoa, hyperactivation, acrosome reaction and sperm-oocyte fusion (Baker, Hetherington, Weinberg and Velkov, 2014; Baker and Aitken, 2005; O'Flaherty, 2015).

In North Macedonia, accurate information regarding this sensitive problem seems to be utopic. This happens because of superstition, illiteracy, social stigma, and shame. An unnecessary reason is a heedlessness by appropriate healthcare providers in noting and encouraging males in seeking medical care for this problem. Even though some unpublished data, and some published for the south-western part of North Macedonia, suggest that male factor infertility in our country is solely responsible in approximately 44.2% of cases (Zhaku, Beadini, Beadini, Xhaferi and Golaboska, 2019).

Since patients with oligoasthenozoospermia are a very diverse group, treating this particular group is not an easy task. Despite the common association between compromised sperm physiology by elevated OS, men in North Macedonia are not screened for OS nor treated for this condition. Researchers believe that the sperm is more susceptible to OS than other cells due to the limited amount of cytoplasm in mature sperm and the concentration of ROS-suppressing antioxidants in the sperm as well as high levels of unsaturated fatty acids in the sperm structure (Saleh and Agarwal, 2002). Therefore the health and fertility of sperm is greatly dependent on the availability of antioxidants which is mostly related to the antioxidant systems in seminal plasma (Nematollah et al., 2017). Seminal plasma is well endowed with an array of antioxidants that act as free radical scavengers. Non-enzymatic antioxidants such as vitamin C, vitamin E, pyruvate, glutathione and carnitines protect spermatozoa against OS. A number of enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase achieve the same purpose (Agarwal and Saleh, 2002; Agarwal, Nallella, Allamaneni and Said, 2004). There are many studies published by serious relevant healthcare institutions that support the use of antioxidant supplements because they augment the scavenging capacity against free radicals and serve as a stabilizer in the homeostasis of spermatogenesis. Antioxidants also provide energy for male germ cells by preserving the intracellular milieu in a reduced state, and they protect these cells from OS (Pryor et al., 2006; Mates and Sanchez-Jimenez, 1999).

Thus, we intend to study the efficacy of antioxidant therapy in the reduction of OS, and the improvement of semen parameters in men with oligozoospermia, asthenozoospermia or oligoasthenozoospermia.

2. Materials and Methods

Study design and eligibility criteria

In this prospective interventional study, 37 men (age group 21-41; mean age, 30.9), were enrolled between March 2017 and November 2018, which were unable to conceive their healthy spouses (one of the eligibility criteria). All infertile patients were married for at least one year. The diagnostic procedure was done after two consecutive semen analyses in ten days interval, at the Department of Physiology and Biochemistry in the Faculty of Medical Sciences, University of Tetovo. All men showing decreased concentrations (>15 mill/ml) and motility of spermatozoa (< 32%), according to the World Health Organization (WHO) guidelines (WHO Lab. Manual, 2010), meet the eligibility criteria for participating in this study. Exclusion

criteria for the male participants were as follows: use of antioxidant agents or vitamins within 8 weeks prior to inclusion in the study, a history of excessive consumption of alcohol 40 days prior to the start of the trial, patients that showed lower than 5% motility and less than 1×10^6/ml sperm concentration, patients with any acute or chronic disease or who are undergoing some kind of treatment with any class of drugs and subjects with known hypersensitivity to ingredients in the antioxidant formula.

All patients took a capsule consisting of 500 mgs of Maca substance, three times a day. The other was a tablet consisted of a combination of 60 mg Korean Ginseng Extract, 100 mg vitamin C, 67 mg vitamin E, 15 mg zinc, 200 μg selenium, 250 mg L-Arginine, 50 mg L-Carnitine, 50 mg L-Methionine and 50 mg L-Phenylalanine, available in North Macedonia under a brand name, manufactured in the United Kingdom, taken 2 times a day. The subject received this dual combination for a period of 6 months.

Sample collection and semen analysis procedure

Samples were obtained by masturbation from all patients in a room beside the laboratory and placed in sterile containers after at least 3 days of sexual abstinence. The containers were closed and labeled according to name, age, time of ejaculate and duration of abstinence. Semen parameters were analyzed within 30-60 minutes after liquefaction of the sample. It was used in a 100-μm-deep improved Neubauer hemocytometer for determining concentration and motility of spermatozoa.

The first semen sample was collected at a time of enrollment in the study. From this 0.5 ml of the sample was used for standard semen analysis while the rest of the semen was centrifuged at a speed of 3,000 rpms at room temperature for a period of 10 minutes in order to separate plasma from sperm. After this procedure 1.5 ml seminal plasma was frozen and kept in order to evaluate levels of OS parameters like Malondialdehyde (MDA) and Protein Carbonyl (PC). The second semen sample was collected 6 months after therapy initiation and followed the same procedure described above.

Measurement of MDA

Seminal plasma, which is a complex mixture secreted from the testes, epididymis and accessory glands, can affect sperm morphology, motility, acrosome reaction and fertility (Mann and Lutwak-Mann, 1981).
MDA is one of many low molecular weight end-products of lipid hydroperoxide decomposition and is most often measured as an index of lipid peroxidation (de Zwart, et al., 1999). The method of accessing MDA is based on its colorimetric reaction with thiobarbituric acid (TBA) reagent. To 0.1 ml of seminal plasma is added 0.9 ml of distilled water. To this component is added 0.5 ml of TBA which forms a pink colored liquid after heating for one hour in boiling bathwater. After this, the sample is left for cooling and later is centrifuged for 10 minutes at 4,000 rpm (Chaudhari, Das, and Singh, 2008). The absorbance of the supernatant is then measured by a spectrophotometer at a wavelength of 534 nm, and the concentration of MDA was expressed in nmol/mL serum (Draper and Hadley, 1990).

Measurement of PC

The formation and accumulation of protein carbonyls is increased in various human diseases such as Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis, cataract-genesis, cystic fibrosis, diabetes and cardiovascular disease, rheumatoid arthritis, etc. (Dalle-Donne, Aldini, Carini, Colombo, Rossi

and Milzani, 2006; Dayanand, Pradeep and Kutty, 2012; Zusterzeel, Mulder, Peters, Wiseman and Steegers, 2000; Ramsay, Demayo, Hegemier, Wearden, Smith and Welty, 2001).

The introduction of carbonyl groups (aldehydes and ketones) into amino acid residues of protein is a hallmark for oxidative modification (Levine, et al., 1990). The assessment of protein carbonyls offers some advantages because it is a marker that occurs in the early stages of pathology. This remains in circulation for a long time compared to other biomarkers of oxidative stress such as malondialdehyde, or 4-hydroxy-2-nonenal or glutathione (Purdel, Margina and Ilie, 2014).

In the last 20 years various analytical methods for the assessment of protein carbonyls were developed and validated. These include spectrophotometric assays, high-performance liquid chromatography with diode-array or fluorescence detectors, enzyme-linked immunosorbent assays (ELISA), one- or two-dimensional electrophoresis and Western Blot immunoassays or capillary electrophoresis with laser-induced fluorescence detectors (Calabrese, et al., 2006; Mantle, Falkous and Walker, 1999; Levine, et al., 1990; Akagawa, et al., 2006).

Protein carbonyls were measured by using the method of Levine et al (1990, 1994). Briefly, 15 ML of seminal plasma was placed in each of the two glass tubes. Then 0.5 ml of 10 mM DNPH in 2.5 M HCI was added to one of the tubes, while 0.5 ml HCI (2.5 mM) was added to the second tube. The tubes were incubated for 1 hour at room temperature. Samples were vortexed every 15 min. Then 0.5 mL TCA (20%, w $v^{-}l$) was added and the tubes were left on ice for 5 minutes. This was followed by centrifugation for 5 minutes to collect the protein precipitates. The pellet was then washed three times with 2 ml of ethanol-ethyl acetate (1 : 1, v $v^{-}l$). The final precipitate was dissolved in 1 ml of guanidine hydrochloride solution (6 M) and was incubated for 15 minutes at 37 ^{0}C while mixing. The absorbance of the sample was measured at 365 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH (e = 2.2xl 0^{4} cm $M^{-}l$) and expressed as nanomoles per milligram of protein.

Statistical analysis

Data were processed with the Statistical Package for Social Sciences (version 16.0 for Windows; SPSS, Inc., Chicago, IL). Normal distribution of data was tested using the Kolmogorov-Smirnov test. Values are expressed as mean ± SD. The Pearson correlation coefficient was used to analyze the relationship between MDA and PC levels with sperm motility and sperm concentration. This was expressed in a linear regression model.

Hypotheses and differences of parameters were tested using the paired t-test and $p<0.05$ was considered statistically significant.

Ethics

The study was conducted in line with European urology and good clinical practice guidelines with ethical principles laid down in the latest version of the Declaration of Helsinki. All patients signed informed written consent and verbal explanations of the nature of the study. The study design was approved by the institutional review board of the Faculty of Medical Sciences of the University of Tetova.

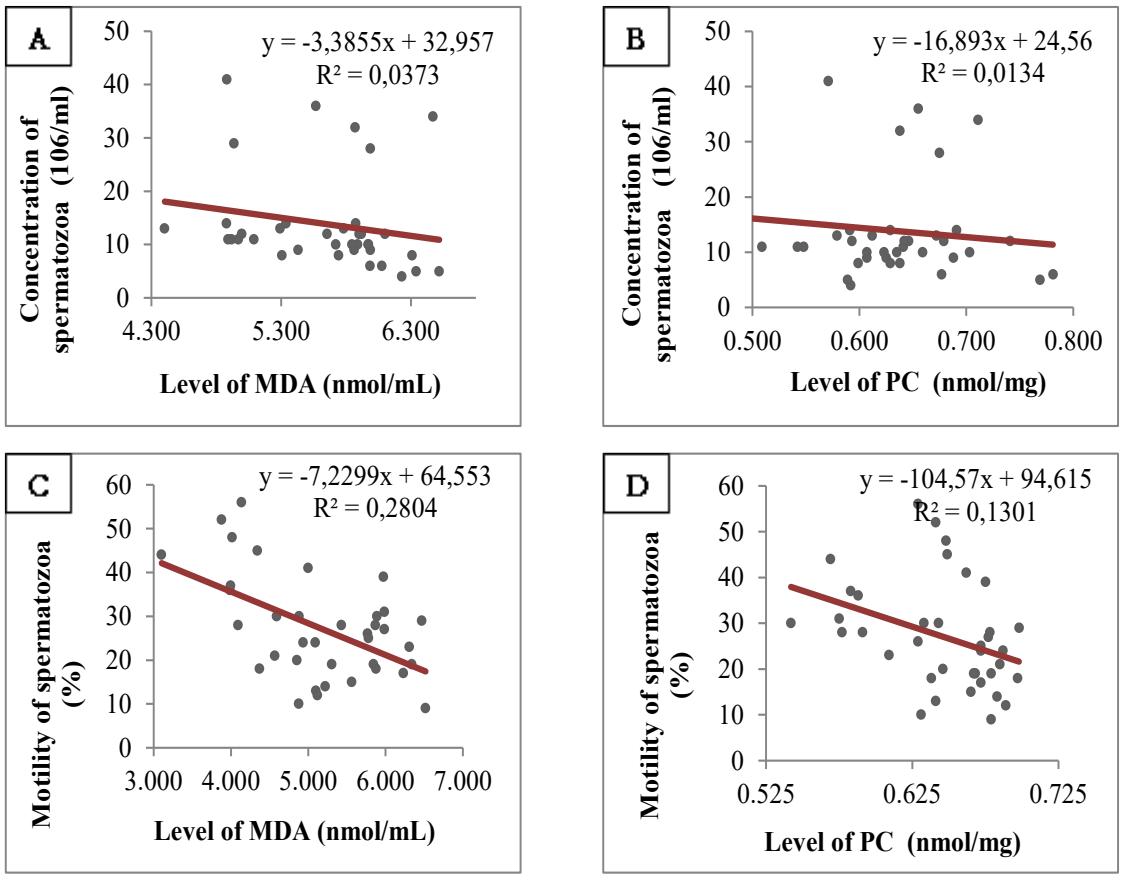
3. Results

Out of 37 infertile males enrolled in the study group 9 patients had oligozoospermia, which means that they had decreased sperm concentration, 6 had asthenozoospermia (decreased sperm motility) and 22 patients had both decreased, sperm concentration and sperm motility, so by that they were classified in the oligoasthenozoospermic group. Noticeable is the fact that all the men included in the study, had increased oxidative stress parameters, elevated MDA and PC levels, which are negatively correlated with sperm concentration and sperm motility (see graphics 1).

Table 1 Semen and OS parameters, presented as mean ± SD, before treatment with antioxidants

Semen profile	Sperm conc. (10^6/ml)	Sperm motility (%)	MDA (nmol/mL)	PC (nmol/mg)
Oligozoospermia (n=9)	11,44 ± 2,13	44,22 ± 6,82	4,270 ± 0,806	0,627 ± 0,038
Asthenozoospermia (n=6)	33,33 ± 4,80	27,16 ± 3,87	5,450 ± 0,758	0,659 ±0,047
Oligoasthenozoospemia (n=22)	9,5 ± 2,86	20,18 ± 6,46	5,460 ± 0,662	0,648 ±0,039
Total (n=37)	13,84 ± 9,23	27,16 ± 11,80	5,172 ± 0,864	0,645 ± 0,041

Data are reported as mean ± SD. N – Number of men included in the study; SD – Standard Deviation; MDA- Malonedialdehyde; PC – Protein Carbonyl



Graph. 1. Correlation between MDA levels with sperm concentration (A), motility (C) and PC levels (B), (D) respectively, before receiving the antioxidant therapy

Levels of MDA are negatively correlated with sperm motility *(r= -0.53)* and sperm concentration *(r= -0.19),* so show and levels of PC with sperm motility *(r= -0.36)* and sperm concentration *(r= -0.12)*. After six months of antioxidant therapy intake, there is a conspicuous increase in the mean of sperm concentration *(p<0.001)*, sperm motility *(p<0.001)*. Also, a decrease in the values of MDA *(p<0.001)*, while a decrease in PC was not statistically significant *(p=0.0554).*

At the end of the treatment 22 males semen parameters were ameliorated and were classified as normozoospermic, while 15 other males showed improvements in semen parameters and decrease OS parameters, but yet they did not meet the WHO criteria and to be classified as normozoospermic. The values of semen and oxidative stress parameters are shown in Table 2.

Table 2. Semen and OS parameters, presented as mean ± SD, after six months of antioxidant therapy

Semen profile	Sperm conc. (10^6/ml)	Sperm motility (%)	MDA (nmol/mL)	PC (nmol/mg)
Oligozoospermia (n=9)	20,67 ± 8,19	46,78 ± 6,59	3,480 ± 0,702	0,615 ± 0,037
Asthenozoospermia (n=6)	34,83 ± 5,12	37,16 ± 1,83	4,090 ± 0,737	0,652 ± 0,049
Oligoasthenozoospemia (n=22)	16,31 ± 5,24	28 ± 6,75	4,130 ± 0,502	0,642 ± 0,040
Total (n=37)	20,38 ± 8,91	34,05 ± 10,06	3,968 ± 0,640	0,637 ± 0,042

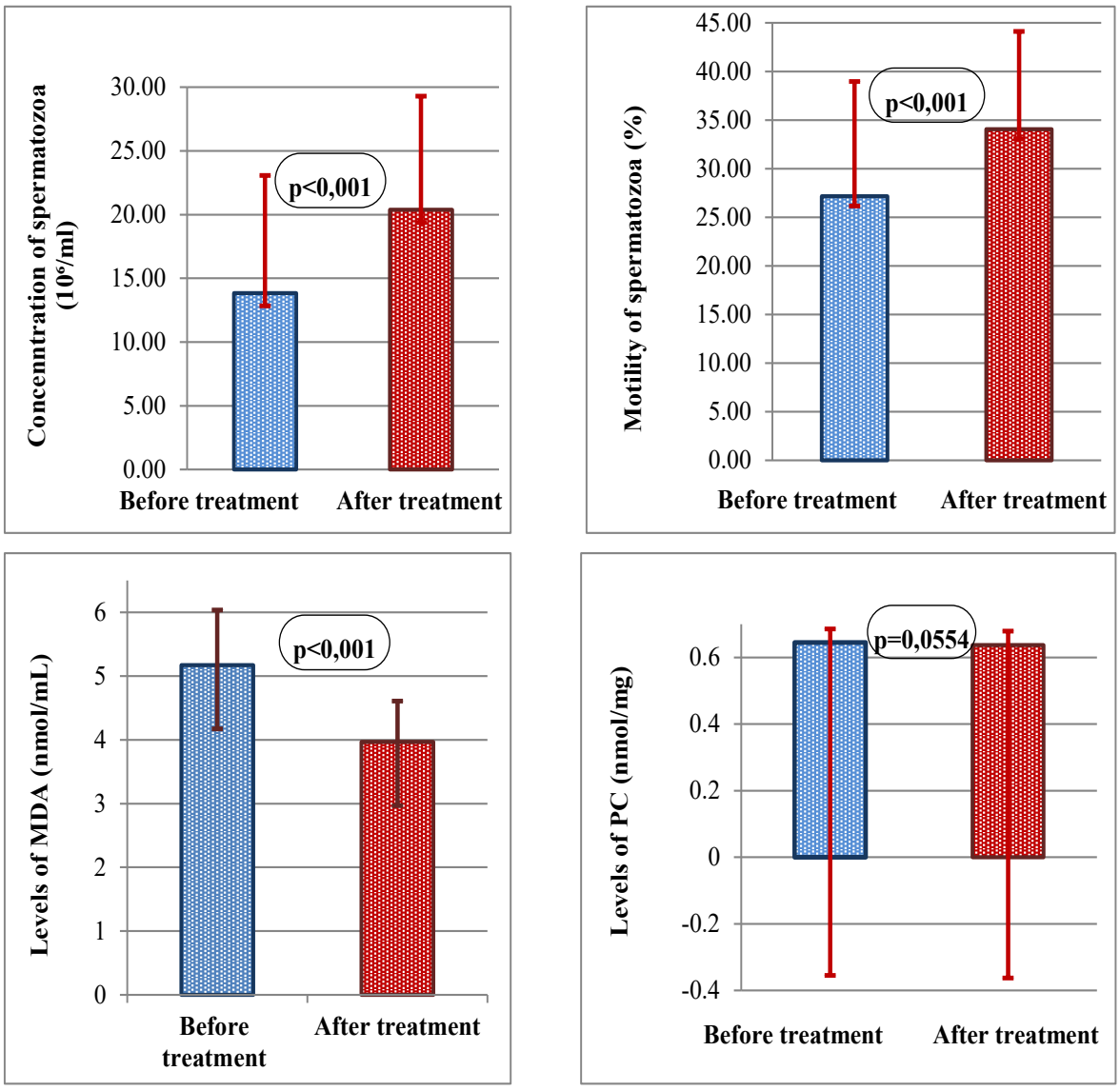
In table 3 are shown differences in sperm concentration, sperm motility, MDA and PC contents for each of the three groups consecutively. Every difference lower than 0.05, was considered statistically significant.

Table 3. Differences of the variables, before and after treatment (p>0,001 is considered statistically significant

Variable	Oligozoospermia		Asthenozoospermia		Oligoasthenozoospermia	
	Difference	*p-value*	***Difference***	*p-value*	***Difference***	*p-value*
Concentration	-9,23 ± 6,06	**p=0,0056***	-1,5 ± 0,32	p=0,404**	-6,81 ± 2,38	**p<0,001***
Motility	-2.56 ± 0,23	p=0,247**	-10 ± 2,04	**p<0,001***	-7,82 ± 0,29	**p<0,001***
MDA	0,79 ± 0,104	**p<0,001***	1,36 ± 0,021	**p=0,0017***	1,33 ± 0,16	**p<0,001***
PC	0,012 ± 0,001	**P=0,0198***	0,007 ± 0,002	p=0,134**	0,006 ± 0,001	**p=0,0178***

***statistically significant**; ** no significance

All group results (of 37 males), showed significant improvement after treatment with the combined antioxidant formula. Significant improvements were seen in a context of sperm motility *(p<0.001),* sperm concentration *(p<0.001)* and significant decrease of MDA *(p<0.001),* while PC showed decrease levels but they were not significant *(p=0.554).* Results are designed graphically and are represented in the graphics below.



Graph.2 Sperm motility (A), concentration (B), MDA (C) and PC (D) levels, before and after antioxidant treatment

4. Discussion and conclusion

Identification of etiology and physio-pathogenic mechanisms allows general practitioners and clinicians to select the optimal treatment and overcome male infertility. Raising awareness about treating options in North Macedonia, while sperm parameters are decreasing because of increased oxidative stress, in a terrain like this, represents a big challenge.

As mentioned in the introduction part, there are a lot of studies (Micic, et al., 2019; Ciftci, Verit, Savas, Yeni and Erel, 2009; Akmal, Qadri, Al-Waili, Thangal, Haq and Saloom, 2006; Kessopoulou, et al., 1995; Badade, More, Narshetty and Badade-Vandana, 2011; Busetto, et al., 2018), that support the use of antioxidants, especially in idiopathic male infertility, as well as there are studies (Raigani, et al., 2014; Comhaire, Christophe, Zalata, Dhooge, Mahmoud and Depuydt, 2000; Safarinejad, Shafiei, and Safarinejad, 2011), that show no benefit in the use of antioxidants in treating infertile men.

In our study, we found that all the infertile males had significantly higher levels of MDA and PC, which suggests that they have a high induced oxidative stress, which impacts sperm concentration and sperm motility (sperm morphology was not the aim of the study).
After antioxidant supplementation, we observed a significant improvement of semen parameters *(p<0.001)* and a decrease of MDA *(p<0.001)* while there was not a significant decrease of PC *(p=0.554)*. Improvements in sperm concentration and motility followed by decrease levels of the oxidative stress marker – MDA had observed also Suleiman, Ali, Zaki, El-Malik and Nasr (1996); Geva E et al., (1996); Eskenazi B et al., (2005); Makker, Agarwal, and Sharma (2009); and Singh, Jahan, Radhakrishnan, and Banerjee, (2016).
In oligozoospermic males included in this study, there was a significant improvement of sperm concentration (p=0.0056) while no significant improvement was seen in sperm motility *(p=0.247)*. MDA and PC levels showed also a significant decrease (*p<0.001* and *p=0.0198* respectively).
The asthenozoospermic group showed significant improvements in sperm motility *(p<0.001)* and a decrease of MDA levels *(p=0.0017)*. No significant improvements were seen in sperm concentration *(p=0.404)* and levels of PC *(p=0.134)*.
Referring to the Oligoasthenoteratozoospermic group, it could be seen that there were significant improvements in sperm motility, concentration, MDA *(p<0.001),* and PC *(p=0.0178)*.
In all three groups, namely, all 37 participating males showed a negative correlation between MDA levels with sperm concentration *(r= -0.19)* and sperm motility *(r= -0.53)*. A negative correlation was found also between PC levels with sperm motility (r= -0.36) while not a strong correlation was seen with sperm concentration *(r= -0.12)*. Our results of MDA are concurrent with Singh, Jahan, Radhakrishnan, and Banerjee, (2016); Masroor, Muneshwar, and Zingade, (2013); Kobayashi, Miyazaki, Natori, and Nozawa, (1991), which demonstrated high seminal MDA levels in patients with sperm motility and concentration below the referent values according to the WHO manual, 2010.

Once a male has been pointed as having oxidative stress related infertility, treatment strategy should comprehend identification, modification and amelioration of the underlying cause before considering antioxidant supplements. Lifestyle behaviors such as smoking, alcohol & drug abuse, excessive use of caffeine, poor vitamin diet, fast food, inactivity, obesity, pollution, radiation, and excess psychological stress have all been linked to increased oxidative stress (Durairajanayagam, 2018; Sharma, et al., 2010; Bhongade, Prasad, Jiloha, Ray, Mohapatra and Koner, 2015). There is, however, lack of agreement, because improvement is not consistent and there is wide variation in the treatment regimens, on the dose and duration of treatment and whether mono or combined oral antioxidants should be administered.
Moreover, antioxidant supplements are not free from potential side effects “antioxidant paradox”, because overuse of antioxidants such as vitamin C, vitamin E, *N*-acetyl cysteine may lead to reductive stress, which is reported to be as dangerous to cells as oxidative stress and can be the cause of diseases such as cancer or cardiomyopathy (Henkel, Sandhu and Agarwal, 2019).
Future directions include identifying the underlying molecular mechanisms that explain the specific effects of some antioxidants on semen parameters (Alahmar, 2018).

5. Limitation of the study

However, the present study has several limitations. First, the number of patients included in the study was small to make proper conclusions. Second, we assessed only oxidative stress markers (MDA and PC), but we didn't test antioxidant capacity (level of Total Antioxidant Capacity – TAC). We haven't included a control group (fertile males) which will also be tested also for MDA and PC levels and their correlation with sperm motility and concentration. Also, the interventional group should be randomizelly selected, treating half of them with placebo therapy and the other half with the selected antioxidant formula, that's why, further placebo-controlled, dietary-controlled, double-blind, randomized - prospective studies with standardized supplement regimens are needed, in order to elucidate the role of antioxidant therapy in the treatment of oxidative stress and management of male infertility.

Nomenclature

OS	Oxidative Stress
ROS	Reactive oxygen species
MDA	Malonedialdehyde
PC	Protein Carbonyl
OA	Oligoasthenozoospermia
DNA	Deoxyribonucleic acid
ASRM	American Society for Reproductive Medicine
OZ	Oligozoospermia
AZ	Asthenozoospermia
OAZ	Oligoasthenozoospermia
WHO	World Health Organization
TBA	Thiobarbituric acid
ELISA	enzyme-linked immunosorbent assays
DNPH	Dinitrophenylhydrazine
HCI	Hydrochloride
TCA	Trichloroacetic acid

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