

Oral Antioxidants Mitigate Levels of Malonedialdehyde and Protein Carbonyl and Improve Semen Parameters in Men with Oligoasthenozoospermia

Vegim Zhaku (✉ vegim.zhaku@unite.edu.mk)

University of Tetova <https://orcid.org/0000-0003-3972-0542>

Sheqibe Beadini

University of Tetova

Nexhbedin Beadini

University of Tetova

Gazmend Amzai

4. University Clininc of Hematology, Clinical Center Mother Theresa Skopje

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Abstract

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.

All the patients that fulfilled the required criteria, 37 were enrolled in the study signing a document that explains nature of the study. The first semen sample was collected with at least three days of abstinence. From the sample 0.5 ml was used for standard semen analysis, 1.2 ml was used to evaluate the levels of malonedialdehyde (MDA) and protein carbonyl (PC). The last sample was collected after 6 months. Mean, Standard Deviation, the Pearson Correlation and an independent student t-test were used for statistical analyses.

Concentration and motility were significantly increased after 6 months of treatment ($p < 0.001$).

The level of MDA shows significantly lower values after six months of therapy with antioxidants ($p < 0.001$). Whereas another marker which is denoted by PC was also lower after the treatment, but was not statistically significant ($p = 0.0554$).

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. Always keep on mind that, antioxidants are not free from potential side effects "antioxidant paradox".

Introduction

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" [1, 2].

The incidence of infertility is approximately 15% [3]. Out of this percentage males and females contribute equally in 50% of cases respectively [4].

Normal reproductive function is very important for self-confidence, worthfulness, respect and sexual satisfaction in males.

In North Macedonia accurate information regarding this sensitive problem is still lacking, a given hand also by social stigma and neglecting institutional support for this issue, that's why for our team this cause represents a big challenge. Some results for the south-western part of North Macedonia also published by our team suggest that male factor infertility is solely responsible in approximately 44.2% of cases [5].

There are several multiple causes that 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% [6-11]. When we say idiopathic we actually mean oxidative stress (OS). This is an imbalance between pro-oxidants and antioxidants [12]. It has been proven in various studies that high levels of ROS are present in unexplained male infertility. Under physiological conditions spermatozoa produces small amounts of ROS, which are needed for capacitation, maturation of spermatozoa, hyper activation, acrosome reaction and sperm-oocyte fusion [13-15].

We have to keep on mind that patients with oligoasthenozoospermia are a very heterogeneous 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 still not screened for OS, so antioxidant treatment is not always included in the treatment of this category. Researchers believe that the sperm is more susceptible to OS than other cells due to the limited amount of cytoplasm in a 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 [16]. 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 [17]. 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 [18, 19]. Antioxidants also provide energy for male germ cells by preserving the intracellular milieu in a reduced state, and they protect these cells from OS [20, 21].

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 stabilizer in the homeostasis of spermatogenesis. Thus, our intention is 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.

Materials And Methods

- *Study design and eligibility criteria*

In this prospective interventional study, 37 patients (age group 21-41; mean age, 30.9), were enrolled between March 2017 and November 2018, unable to conceive their healthy spouses (one of the eligibility criteria's). All infertile patients were married for at least one year. Diagnostic procedure was done after two consecutive semen analyses at ten day intervals, in ten days interval, at the department of Physiology and Biochemistry by the Faculty of Medical Sciences, University of Tetova. 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 underwent two combinations of antioxidant therapy. One was a capsule consisting of 500 mgs of Maca substance taken 3 times a day, while the other tablet was a combination of 60 mg Korean Ginseng Extract, 100 mg vitamin C, 67 mg vitamin E, 15 mg zinc, 200 mg 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 United Kingdom, taken 2 times a day. This kind of therapy has shown impressive improvements of semen parameters, and decreasing levels of oxidative stress. Subject received this two tablet 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-mm-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 Malonaldehyde (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 [27].

MDA is one of many low molecular weight end-products of lipid hydro peroxide decomposition, and is most often measured as an index of lipid peroxidation [28].

The method of accessing MDA is based on its colorimetric reaction with a 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 bath water. After this the sample is left for cooling, and later is centrifuged for 10 minutes at 4,000 rpm [29]. 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 [30].

- *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 [31-34].

Introduction of carbonyl groups (aldehydes and ketones) into amino acid residues of protein is a hallmark for oxidative modification [35]. 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 [36]. 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 [37-40].

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 HCl was added to one of the tubes, while 0.5 ml HCl (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⁻¹) 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⁻¹). The final precipitate was dissolved in 1 ml of guanidine hydrochloride solution (6 M) and was incubated for 15 minutes at 37 °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 ($\epsilon = 2.2 \times 10^4 \text{ cm M}^{-1}$) and expressed as nanomoles per milligram of protein.

- *Statistical analysis*

The collected data was processed with ANOVA. Values are expressed as mean \pm 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 were tested using the paired t-test. $P < 0.05$ 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 an 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 by the University of Tetova.

Results

Out of 37 infertile males enrolled in the study group 9 patients had oligozoospermia, 6 had asthenozoospermia and 22 patients had oligoasthenozoospermia. According to their parameters some had decreased sperm concentration, some decreased sperm motility or both parameters decreased. Noticeable is the fact that all the men had increased oxidative stress parameters, elevated MDA and PC levels, which are negatively correlated with sperm concentration and sperm motility (see graphics below). Data are presented as mean \pm SD in table 1.

Table 1 Semen and OS parameters, presented as mean \pm 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 \pm 2,13	44,22 \pm 6,82	4,270 \pm 0,806	0,627 \pm 0,038
Asthenozoospermia (n=6)	33,33 \pm 4,80	27,16 \pm 3,87	5,450 \pm 0,758	0,659 \pm 0,047
Oligoasthenozoospermia (n=22)	9,5 \pm 2,86	20,18 \pm 6,46	5,460 \pm 0,662	0,648 \pm 0,039
Total (n=37)	13,84 \pm 9,23	27,16 \pm 11,80	5,172 \pm 0,864	0,645 \pm 0,041

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

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 of OS parameters, but yet they did not meet the WHO criteria and to be classified as normozoospermic. 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
Oligoasthenozoospermia (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	
	<i>Difference</i>	<i>p-value</i>	<i>Difference</i>	<i>p-value</i>	<i>Difference</i>	<i>p-value</i>
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.

Discussion

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.

There are a lot of studies in recent years that support the use of antioxidants, especially in idiopathic male infertility [41-46], as well as there are studies that show no benefit in the use of antioxidants [47-49].

In our study we found that all the infertile males had significant higher levels of MDA and PC, which suggest 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 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 SA et al., Geva E et al., Eskenazi B et al., Makker et al. and Alpana Singh et al. [50, 55-58].

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 significant decrease ($p < 0.001$ and $p = 0.0198$ respectively).

The asthenozoospermic group showed significant improvements in sperm motility ($p < 0.001$) and 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 of sperm motility, concentration, MDA ($p < 0.001$) and PC ($p = 0.0178$).

In all three groups, namely all 37 participating males showed negative correlation between MDA levels with sperm concentration ($r = -0.19$) and sperm motility ($r = -0.53$). Negative correlation was found also between PC levels with sperm motility ($r = -0.36$) while not a strong correlation was seen with sperm concentration ($r = -0.12$). Significant negative correlation was found between seminal MDA levels and sperm motility ($r = -0.39$). Our results of MDA are concurrent with Alpana Singh et al., Shazria et al., Kobayashi et al. [50-52], which demonstrated high seminal MDA levels in patients with sperm motility and concentration below the referent values according to the WHO manual, 2010. 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). Third, Pregnancy rate was not objective of this investigation.

Future directions include identifying the underlying molecular mechanisms that explain the specific effects of some antioxidants on semen parameters [53]. 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 psychological stress have all been linked to increased oxidative stress.

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”. Further placebo-controlled, dietary-controlled, double-blind, randomized-controlled, prospective studies with standardized supplement regimens are needed to elucidate the role of antioxidant therapy in the treatment of oxidative stress and management of male infertility [54].

Declarations

Competing Interests

The authors declare no competing interests.

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 an 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 by the University of Tetova.

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Figures

Correlation between MDA levels with sperm concentration (B), motility (D) and PC levels respectively (A, C), in oligoasthenozoospermic men.

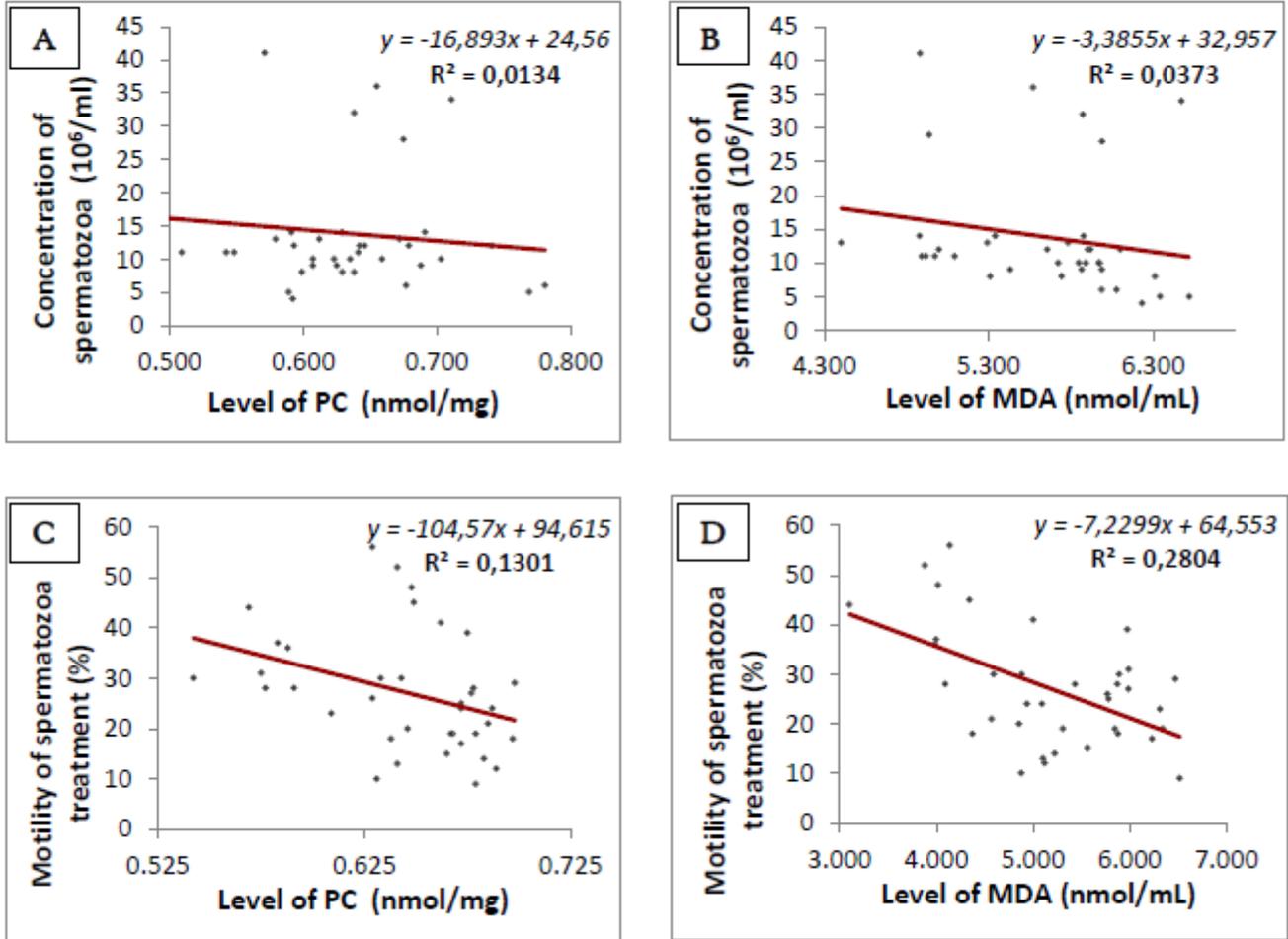


Figure 1

Correlation between MDA levels with sperm concentration (top right) (B), motility (bottom right)(D) and PC levels respectively (A(top left), C(bottom left)), in oligoasthenozoospermic men.

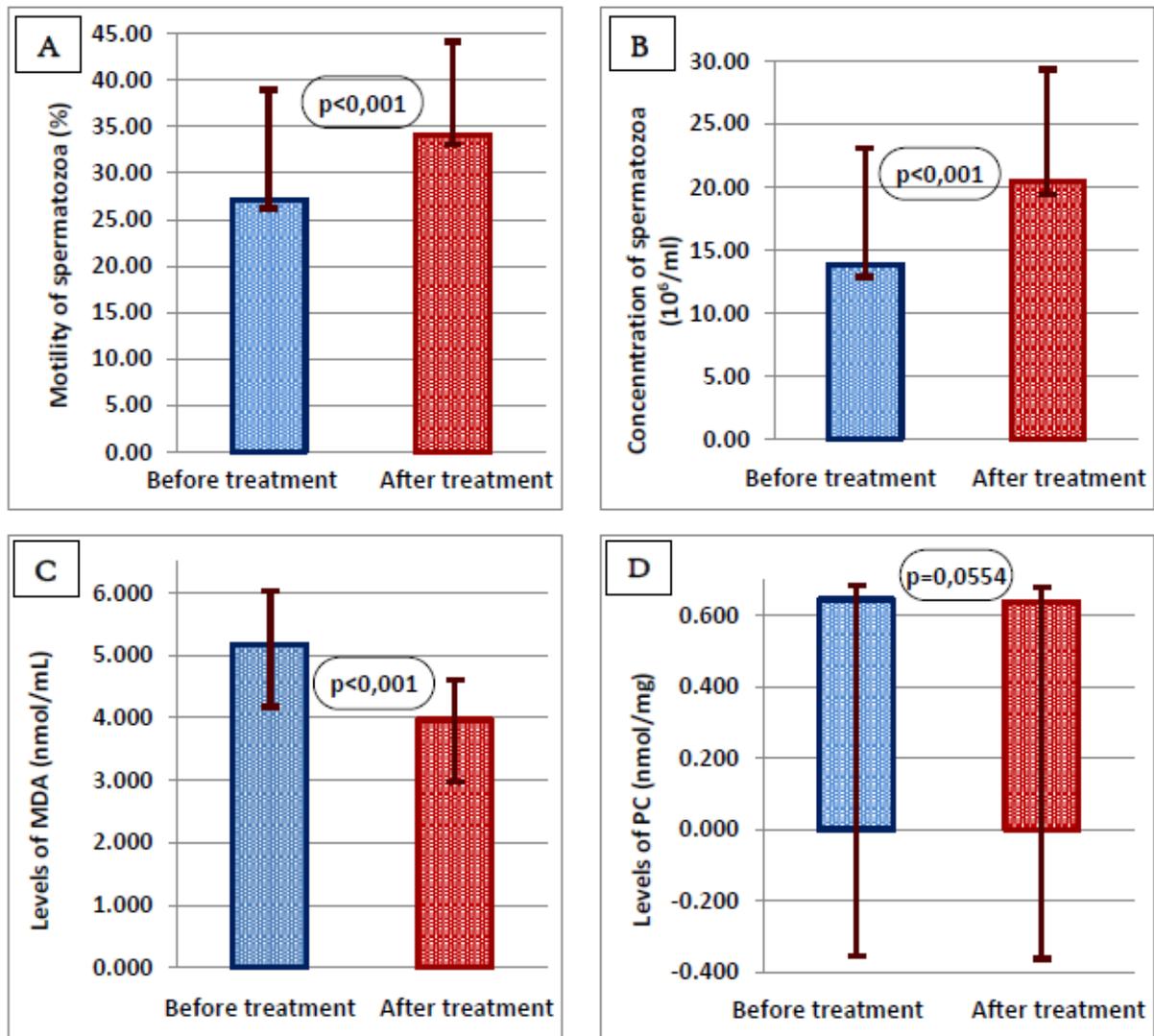


Figure 2

The graphics show the results of sperm motility (Top left)(A), concentration (top right)(B), MDA (bottom left) (C) and PC (bottom right)(D) levels, before and after antioxidant treatment