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**Original scientific paper**

# EFFICACY AND RATIONALE BEHIND COMBINATION OF THE ANTIOXIDANT THERAPY IN MEN WITH OLIGOASTENOZOOSPERMIA AND ELEVATED CASPASE-3 ACTIVITY

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**Abstract**

The rate of male infertility globally without exception has attracted the attention of healthcare professionals and pharmaceutical companies.
To verify the effectiveness of combination therapy with antioxidants as recommended in some elaborated papers in major medical libraries such as PubMed, Cochrane, Scopus, and Elsevier, a total of 57 individuals were enrolled in the study after fulfilling the inclusion criteria. Standard semen analysis along with biochemical analysis of Caspase-3 levels was done before receiving therapy and after six months. The groups were randomly divided into the individuals who received the combination of the antioxidant formula (n=34) and the individuals who received placebo therapy (n=23).
After 6 months of treatment, there was an improvement in concentration (mean & SD, 31.26 ± 3.39) and motility (46.18 ± 5.97) in the group that had received antioxidant therapy for 6 months with statistically significant difference ($p<0.001$), whereas the placebo group showed no statistically significant improvement after
the 6 months in concentration (9.96 ± 3.23; $p=0.950$) nor in motility (30.52 ± -5.29; $p=103$). In the group treated with antioxidants, we had a reduction in caspase-3 activity (2.21 ± 0.62; $p <0.001$), whereas in the placebo group the mean caspase-3 activity had no statistically significant changes (4.05 ± 0.73; $p=0.275$).
Such therapy seeing the cost-benefit of the same in situations when the consequence of male infertility is increased free radical activity is justified and contributes positively to the process of conceiving natural fertilization.

*Keywords:* Male Infertility, antioxidants, caspase-3, fertilization

## 1. Introduction

Living in the famous COVID-19 period, the virus that broke out in Wuhan, China, has been a challenge for all medical professionals both in terms of surviving the deadly agent, but also in terms of follow-up of patients with other comorbidities, facing difficulties in diagnosing and adequate treating.

At the moment of speaking there have been close to 529,4 million reported cases of COVID-19 globally, including approximately 6,3 million deaths, with almost 12 billion vaccine doses administered, as of 6th June 2022 (https://covid19.who.int/). While in North Macedonia there have been 312,157 confirmed cases of COVID-19 with 9,309 deaths and 1,8 million vaccine doses have been administered as of 6th June 2022 (https://covid19.who.int/). Notably, this infection is characterized by a broad spectrum of clinical presentations and affects multiple body organs (Zaim *et al.* 2020). The SARS-CoV-2 enters host cells by binding to membrane angiotensin-converting enzyme II (ACE2) receptors, receptors which are distributed also in the reproductive organs as in: testicular cells, including spermatogonia, Sertoli and Leydig cells, and prostate cells (Bian and Li 2021; Wang and Xu 2020; Chen *et al.* 2020). But, our intention in this paper is not to evaluate the impact of SARS-CoV-2 on the reproductive system, but the impact of global pandemic in

people that face out fertility problems and the continuity in evaluating properly people showing up with this problem in this period of time. Surely a study with the implication of SARS-CoV-2, will remain in another option, after a clearly defined design and methodology related to this issue.

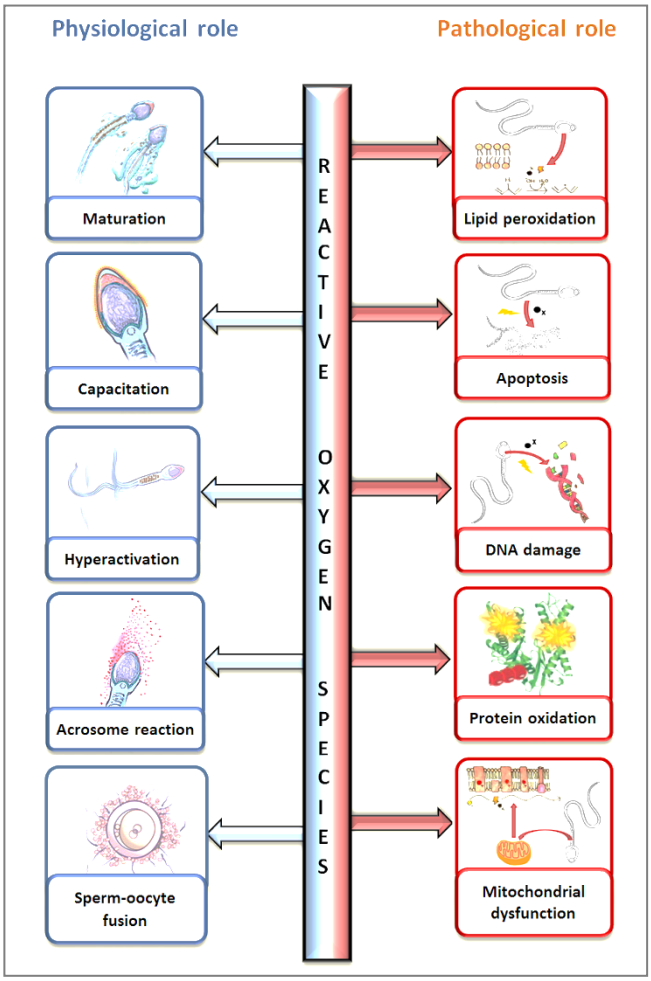



**Figure no.1.** Physiological and patholog ical conse-quences of ROS. ROS dose is a critical parameter in determining the ultimate cellular response, low (nec e ssary) dose for physiological processes and high

Of course, the pandemic had a profound effect in deepening the problem of male infertility worldwide and in North Macedonia also. Based on preliminary data from two studies conducted by our faculty, it revealed that in the region of western Macedonia consisting of 2 regions (Polog and southwestern region), the prevalence of male infertility is around 44.2 ± 3.6% (Beadini et al. 2014; Zhaku et al. 2019).

One of the factors that contribute to male infertility in 32.6% of cases, considered idiopathic etiology is oxidative stress (Naz, Mamuna, and Mehnaz Kamal, 2017). Under physiological conditions, high levels of ROS are counter-balanced by antioxidants, which competently maintain a delicate redox balance by donating their electrons to the ROS and thus interrupting further intake of electrons from surrounding compartments (Alahmar AT, 2018). Several antioxidant compounds are currently prescribed without any scientific rationale, ensuing neither semen parameters improvement, nor fertilization outcomes.

Contrary, some other studies even showed a worsening of semen parameters (Menezo YJ, 2007), because an excess intake of antioxidants can contribute to the establishment of reductive stress (RS), a condition which has been reported being as harmful as OS (Castagne V, 1999), because physiological levels of ROS are responsible for important processes as well: (1) maturation, (2) capacitation, (3) hyperactivation, (4) acrosome reaction and (5) sperm-oocyte fusion (Agarwal A et al, 2008). Physiological and pathological effects as a result of ROS levels are shown in figure no.1.

## 2. Aim of the study

We intend to identify factors that could potentially be markers of increased levels of oxidative stress by measuring caspase 3 (Cas-3) levels. Caspases are proteolytic enzymes that contain cysteine at their active sites, playing a key role in the induction of cell death by cleaving specific proteins in the cytoplasm and nucleus (Li F et al., 2015). Principally, there are two major pathways in the induction of programmed cell death mechanism including the extrinsic pathway (FasL, Fas mechanism, defined by caspase-8) and the intrinsic or mitochondrial pathway (Riedl SJ, and Shi Y, 2014). Caspase-8 cleaves the compartments of the cell directly and also activates the caspase-3 protease effector, which in turn activates other substrates either

directly or indirectly in proceeding apoptosis. On the other hand, the intrinsic pathway is associated with the activation of proteins such as Bcl-2 associated X (Bax) causing mitochondrial disruption and then release of pro-apoptotic mitochondrial factors such as Cytochrome-c which interacts with apoptotic protease activating factor-1(Apaf-1) and activates caspase-9, which in turn activates caspase-3 down-stream (Weng SL et al., 2002). The proteolytic caspase-3 enzyme is considered a central effector of caspase, becoming active at relatively late phases and merging both the extrinsic and intrinsic pathways of apoptosis. The major importance of caspase-3 stimulated us to analyze its levels in infertile men with idiopathic etiology.
Another aim was to observe the correlation between these levels, before and after treatment with the antioxidant combination and the impact of the therapy used in improving concentration and motility in men with oligoasthenozoospermia.

## 3. Material and methods

*Study design and analysis procedure*

In this randomized one-blind placebo-controlled trial, 57 men (age group 19-45; mean age 28,3 ±5,16), were enrolled between March 2017 and April 2021.
All patients who met the criteria to participate in the study were given a clear and understandable verbal explanation and written consent regarding the nature of the study. Every patient was then asked to sign the acceptance form of participation as their biological material and personal data will be used for this study.
The diagnostic procedure was done after two consecutive semen analyses in ten days intervals, at the department of Physiology and Biochemistry in the Faculty of Medical Sciences, University of Tetova. The first semen sample was collected at the time of enrollment in the study. From this 1 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 10 minutes to separate plasma from sperm. After this procedure 1.5 ml seminal plasma was frozen and kept to evaluate the caspase-3 levels. The second semen sample was collected 6 months after therapy initiation and followed the same procedure described above.
The 57 men who were eligible to participate in the study were randomly divided into two groups: the group that would receive the combination with antioxidants (n=34) and the group that would receive a placebo (n=23).
The antioxidant formula consisted of 500 mgs of Maca substance, three times a day, and a tablet consisted of 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 the United Kingdom, taken 2 times a day. The subject received this dual combination for 6 months. The placebo group received the same-looking therapy, without active ingredients on it.

*Eligibility criteria*

Men, which were unable to conceive their healthy spouses, after one year of regular, unprotected sexual intercourse; all infertile patients were married for at least one year. 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; Patients which showed the normal structure of the urogenital tract; men without psychosexual and ejaculatory dysfunction. Exclusion criteria for the male participants were as follows: use of antioxidant agents or vitamins within 8 weeks before inclusion in the study; a history of excessive consumption of alcohol 40 days before the start of the trial; patients that showed lower than 5% motility and less than 1 ×

106/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.

*Measurement of Caspase-3*

The colorimetric method was used as it is based on a simple and convenient analysis evaluating Cas-3 activity in DEVD sequence recognition. DEVD is a Cas-3-specific sequence that is bound to a fluorophore DNA molecule. This substrate can freely diffuse along the cell membrane and when inserted into the apoptotic cell, Cas-3 (but also other groups in this family) investigate and cleave the DEVD sequence by releasing the DNA probe. When the probe is inserted inside the nucleus it binds to the DNA of the nucleus producing a light green fluorescent signal.
The Caspase-3 / CPP32 colorimetric analysis kit with a capacity of 100 assays was used to measure Cas-3 activation levels (optical density (OD) 405 nm). The analysis package contains the following components: 2x reactive buffer (5 ml), cell lysis buffer (15 ml), DEVD-pNA (0.5 ml), DTT (0.15 ml). The Cas-3 analysis protocol is based on the formation of the p-nitroaniline chromophore (p-NA) by separation from the substrate labeled DEVD-pNA.

*Statistical analysis*

Data were processed with the Statistical Package for Social Sciences (version 16.0 for Windows; SPSS, Inc., Chicago, IL). The normal distribution of data was tested using the Kolmogorov-Smirnov test. Values are expressed as mean ± SD. Since the distribution was not normal, non-parametric tests were used. The Spearman correlation coefficient was used to analyze the relationship between Cas-3 with sperm motility and sperm concentration.
Differences in parameters pre and post-treatment were tested using the Mann-Whitney U test and $p<0.001$ 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. This study was approved by the institutional board authorized for the evaluation of doctoral theses of the University of Tetova, decision number 22-63/3. Also, this study is registered at www.clinicaltrials.gov, a resource provided by the U.S National Library of Medicine - National Institutes of Health (NIH) with *ID: NCT05135143*.

## 4. Results

The 57 individuals who met the criteria to enter the study and receive such a combination, have been followed over nearly 50 months, because it is technically and practically impossible for the whole group to track at the same time, so the data is received and processed within this period. It should also be noted that the number of individuals approved to be admitted to the study was 68, but 3 individuals from the group receiving antioxidant therapy and 8 individuals from the group with placebo discontinued the study, ie did not a complete treatment or did not show up to analyze the changes of semen profile and oxidative stress levels.

Table 1 presents the levels of concentration, motility, and caspase 3 in both phases (T0 – before starting the

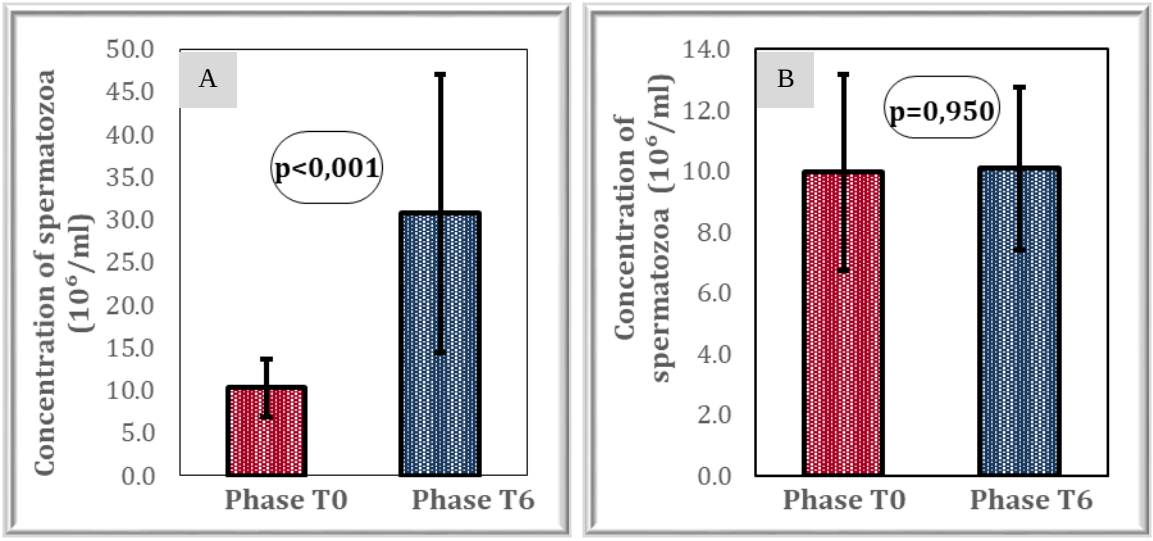
therapy and T6 after six months of receiving the therapy), for both groups included in the study (the antioxidant group and the placebo-receiving group). Details are presented in the form of a mean and standard deviation below.

***Table no. 1*** Differences between the grous treated with antioxidant formula and those treated with placebo in two points (T0 and T6)

| ***Parameters*** | **Antioxidant group** | | | **Placebo group** | | |
|---|---|---|---|---|---|---|
| | **$T_0$** | **$T_6$** | **p-value*** | **$T_0$** | **$T_6$** | **p-value*** |
| ***Concentration(x $10^6$ mmol/L)*** | 10,19±4,11 | 31,26 ±3,39 | p<0,001 | 9,91±3,79 | 9,96±3,23 | p=0,950 |
| ***Motility (a+b %)*** | 31,38±5,71 | 46,18±5,97 | p<0,001 | 29,82±6,03 | 30,52± 5,29 | p=0,103 |
| ***Cas-3 (ng/mL)*** | 3,85±0,51 | 2,21±0,62 | p<0,001 | 4,08±0,70 | 4,05±0,73 | p=0,275 |

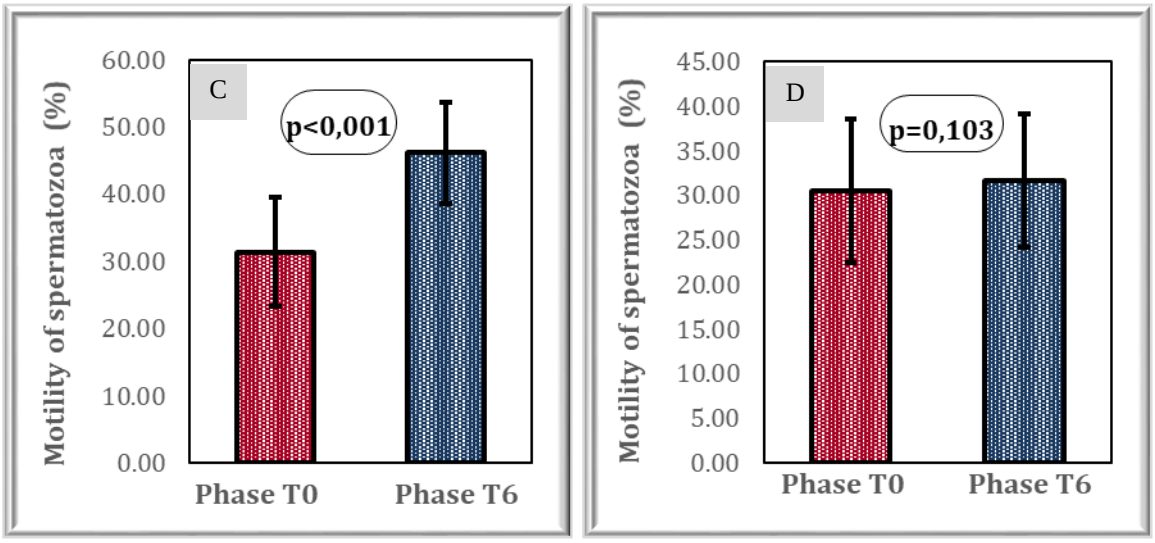
**p<0.001was considered statistically significant. T – refers to time.*

From the table above it can be seen that the group which received the combination with antioxidant therapy, has a significant improvement in terms of concentration (from 10.19±4.11 to 31.26±3.39), a change which in statistical calculations is presented as statistically significant (p<0.001). Even within sperm motility, in the group which was treated with the above combination the changes were statistically significant (from 10.19±4.11 to 31.26±3.39; p <0.001).



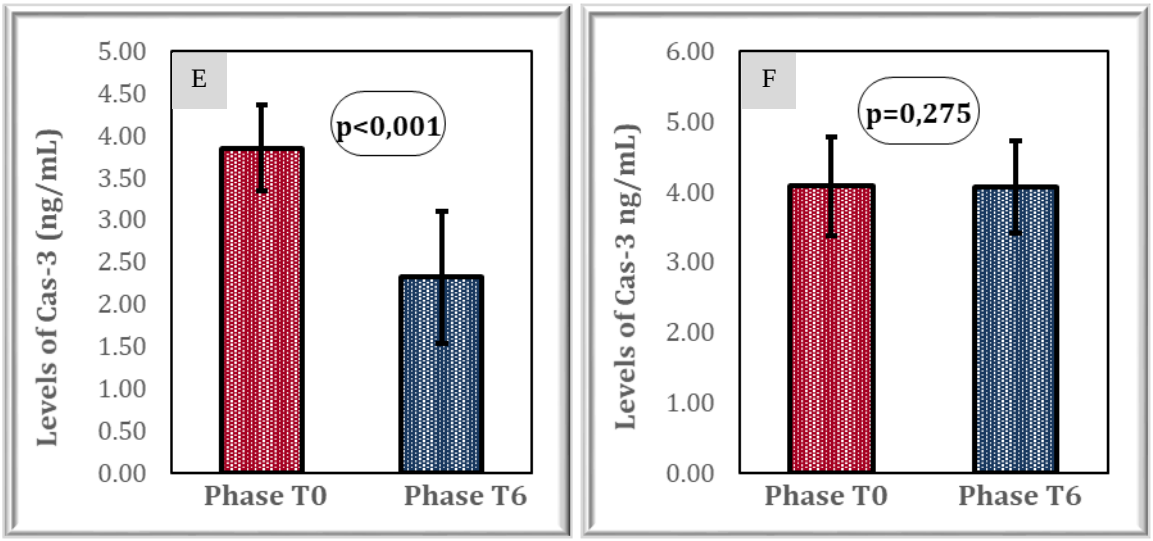

**Graph. No.1** Differences in sperm concentration at T0 and T6 phase in the group receiving the **A.antioxidant formula** and the **B.placebo group.**

Caspase-3 levels were also reduced in the group which received antioxidant treatment (from 3.85±0.51 to 2.21±0.62), a change that was also assessed as statistically significant (p <0.001).




**Graph. No.2** Differences in sperm motility at T0 and T6 phase in the group receiving the **C.antioxidant formula** and the D.placebo group.

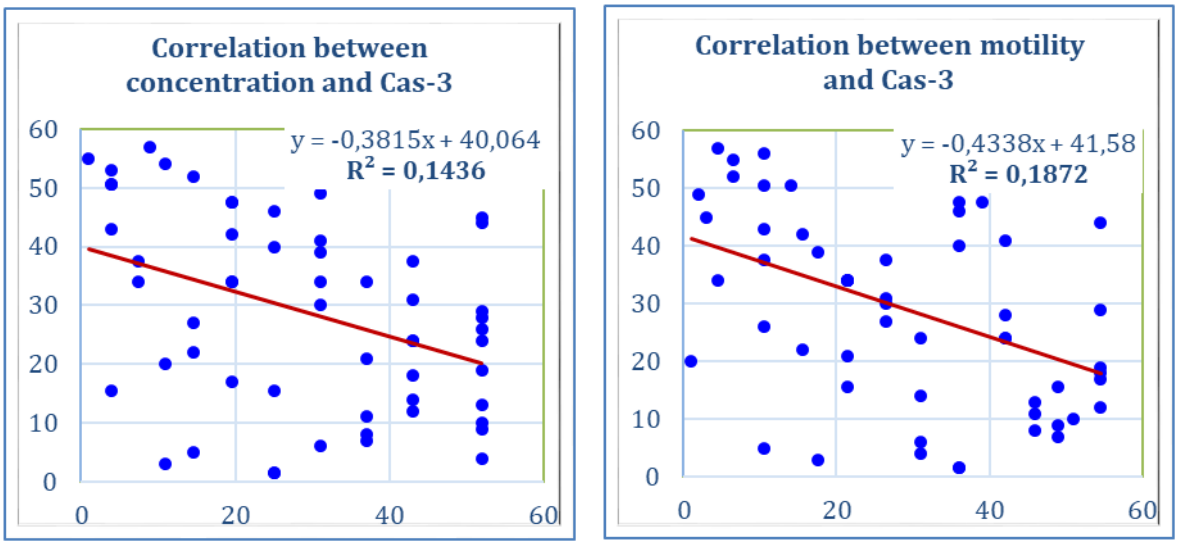
Whereas in the group which during 6 months was treated with placebo therapy no changes were observed in terms of concentration (from 9.91±3.79 to 9.96±3.23; p=0.950), of motility (from 29.82±6.03 to 30.52±5.29; p=0.950) but also that of Caspase-3 (from 4.08±0.70, to 4.05±0.73; p=0.275 ).




**Graph. No.3** Differences in Cas-3 levels at T0 and T6 phase in the group receiving the **E.antioxidant formula** and the **F.placebo group.**

The changes in sperm parameters (concentration and motility), as well as caspase-3 in the antioxidant group and the placebo group, are presented in graphics no.1, 2, and 3.

One of the aims of this study was also to verify whether increased values of caspase-3 correlate with sperm concentration and motility levels in men with oligoasthenozoospermia. The Spearman rank test was used to perform this analysis. Our data resulted that there is a negative correlation between caspase-3 levels and concentration (r=-0.38) as well as motility (r=-0.42). The results are presented graphically in the graph. No.4.




**Graph. No.4** Spearman rank correlation between sperm concentration, sperm motility and Cas-3, for both groups.

## 5. Discussion and conclusion

Reproductive health is a fundamental marker for the society and culture of a nation. Both high and low fertility rates can be problematic for a country's economy, social, political, labor force, and health system.
Recognition pf pathophysiological mechanisms allows medical doctors and policymakers to find out solutions and overcome male infertility and its economicall burden.
Although in the last 3 decades numerous studies have been done on the effect of antioxidants and their role in the treatment of male infertility, it can be concluded that only a small number of studies which meet the criteria for genuine scientific research and publication have reported the positive effects and use of antioxidants in the treatment of male infertility (Comhaire FH et al, 2000; Lenzi A et al, 2003-2004; Ajayi R et al, 2013; Gual-Frau J et al, 2015; Kobori Y et al, 2015; Chattopadhyay R et al, 2016; Stenqvist A et al, 2018; Micic S et al, 2019, Kızılay F et al, 2019 and Illiano E et al, 2020). At the same time, studies that show no benefit from using the combination and certain amounts of antioxidant doses in the treatment of male infertility have been reported controversially (Raigani, et al., 2014; Safarinejad, Shafiei and Safarinejad, 2011).
The scientific reason that defines the combined cocktail of antioxidants is guided by the level of evidence that has been studied for each component in detail. The level of evidence (LE) is based on and modified by the Oxford Center for Evidence-Based Medicine, "The Oxford 2011 Levels of Evidence". (http://www.cebm.net/index.aspx?o=5653).
Most of the components used in this combination are evidence level B, which means that it is based on well-designed studies (prospective and cohort studies) and randomized controlled trials with slightly lower quality than those of level A. What was the effect of each component used on the sperm it is shown figuratively in figure no.2.

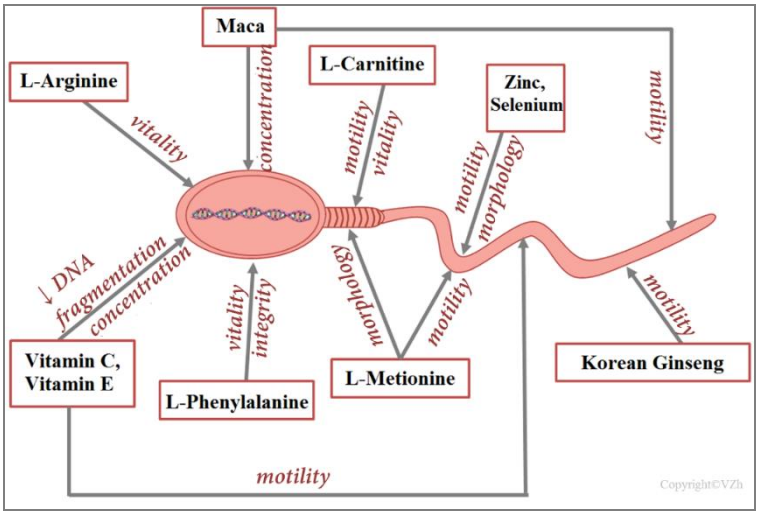



**Figure no.2.** The effect of each component of the used antioxidant combination on spermatozoa. All rights reserved © copyright.

In our study, we found that both groups had significantly higher levels of Caspase-3, which suggest that there is a high induced oxidative stress, which has a detrimental effect on the concentration and motility of spermatozoa, showing a negative correlation, r=-0.38, and r=-0.42 respectively.

After six months of the antioxidant combination, it resulted in a significant improvement in sperm concentration ($p<0.001$), motility ($p<0.001$), and lower caspase-3 levels ($p<0.001$). While in the placebo group there was no significant improvement in the aforemention parameters. Our results are concurrent with the results of similar studies conducted by Micic et al, Lenzi et al., Suleiman et al., Vezina et al., Cheah and Yang, and Balercia et al.

Once a male has been pointed out as having oxidative stress-related infertility, the treatment strategy should comprehend the identification, modification, and amelioration of the underlying cause before considering antioxidant supplements (Agarwal et al, 2004; Zhaku et al. 2020). Bad 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).

However, lack of agreement stills because improvement is not consistent and there is wide variation in the treatment regimens, the dose, and duration of treatment, which in turn intrigues us for further quality and wider sample size studies.

## Nomenclature

| | |
|---|---|
| OS | Oxidative Stress |
| RS | Reductive Stress |
| ROS | Reactive oxygen species |
| Cas-3 | Caspase-3 |
| COVID-19 | Coronavirus Disease 2019 |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| DNA | Deoxyribonucleic acid |
| ACE 2 | angiotensin-converting enzyme II |
| Fas, FasL | pathway for inducing apoptosis, Fas Ligand |

| | |
|---|---|
| Bax | Bcl-2 associated X |
| DEVD | Peptide sewuence for Cas-3 cleavage |
| pNA | p-nitroaniline chormophore |
| DTT | Dithiothreitol |
| OD | Optical Density |
| WHO | World Health Organization |

## Conflict of Interests

All authors declare that there is no conflict of interest regarding the publication of this paper.

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