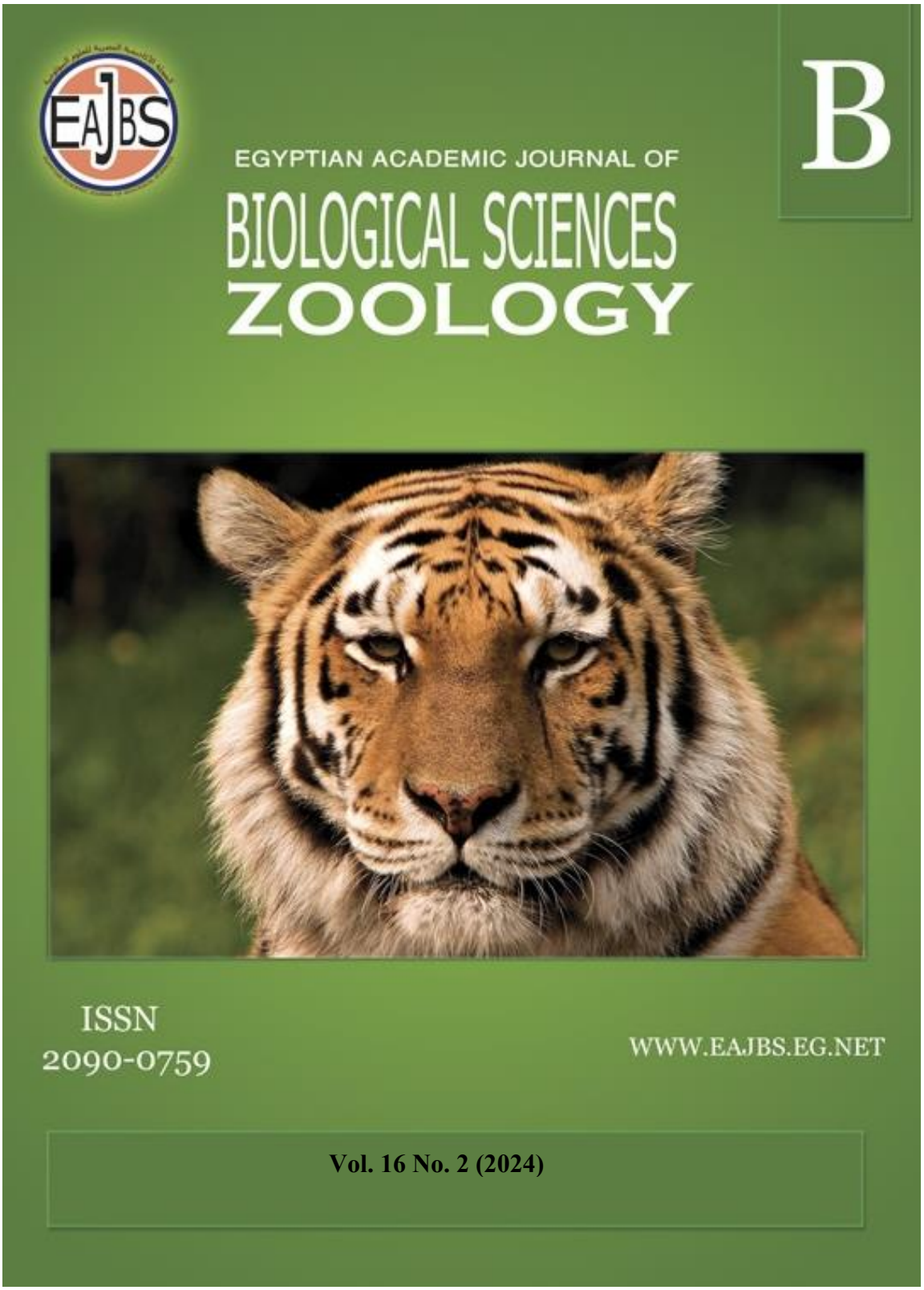

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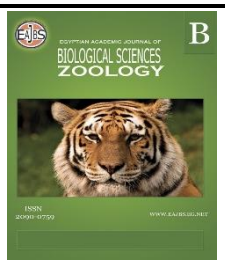

Efficacy of Oral Antioxidant Supplements in Improving Intracytoplasmic Sperm Injection Success Rates

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ABSTRACT

Background: Scientific research has shown that infertile males exhibit more pronounced impairment in sperm quality and higher levels of oxidative stress. Elevated levels of reactive oxygen species have a detrimental effect on the rigidity, motility, and DNA repair capacity of sperm. It is noteworthy that the levels of reactive oxygen species in the body have a significant impact on male fertility, specifically in relation to acrosome response and activation. **Methods:** This study comprised two groups, each comprising 50 couples who were experiencing infertility. Group 1: couples who were unable to conceive due to male-factor infertility. Group 2: couples who are facing infertility, with smoking and the participation of the male partner being identified as contributing factors. Before undergoing intracytoplasmic sperm injection, all male partners received antioxidant therapy in the form of FERTITONE X pills. The study participants provided seminal fluid samples at the beginning of the experiment and then again after a period of three months. The evaluated metrics encompassed semen parameters, including sperm count, motility, viability, morphology, and DNA fragmentation index. **Results:** Administering FERTITONE X treatments has been observed to improve various sperm parameters, such as count, motility, and viability ($p < 0.001$). Furthermore, enhancements in sperm morphology and DNA fragmentation index have been noted across all groups ($p < 0.001$). **Conclusion:** In conclusion, antioxidant therapy improves semen parameters, as well as its influence on the results of in vitro fertilization and intracytoplasmic sperm injection procedures.

INTRODUCTION

The prevalence of infertility varies significantly on a global scale, ranging from 3% to 30%, depending on the specific definition and demographic factors (Keiding *et al.*, 2021). The ability to conceive and become a parent can be influenced by alterations in diet, sleep apnea, hormonal or metabolic disorders, living a high-stress lifestyle, or other medical conditions (Leisegang and Dutta, 2020). Moreover, the correlation between sperm DNA fragmentation and elevated oxidative stress (OS) in idiopathic oligoasthenozoospermia is not fully established. Infertility impacts around 50% of couples

globally, with a prevalence rate of 8-12% among couples (Aitken, 2020).

There is a growing body of evidence indicating that infertility has become more prevalent in recent decades. According to Hussein *et al*. (2021), approximately 15% of couples worldwide face challenges in conceiving a child. Approximately 50% of cases are ascribed to male factors, while 20% are attributed to commonly occurring female variables (Illiano *et al*., 2020). According to Harrison *et al*. (2021), the main factors contributing to male infertility are alterations in sperm motility and morphology, low or absent sperm counts, and changes in sperm ejection. Several studies have demonstrated that various lifestyle factors, such as diet, consumption of fast food, caffeine, smoking, alcohol consumption, obesity, and illicit drug use, have a detrimental effect on male fertility (Leisegang & Dutta, 2020).

Extensive research has been conducted on the correlation between obesity and male fertility, which has revealed detrimental effects on multiple aspects of sperm quality, such as count, motility, and morphology. This aligns with the widely accepted knowledge that obesity in women can have negative consequences on pregnancy outcomes and the health of the fetus (Hernáez *et al*., 2021).

On a global scale, the prevalence of tobacco consumption remains substantial. The numerical data is concerning due to the consistent evidence suggesting a correlation between tobacco smoking and various chronic ailments and negative health outcomes, potentially including carcinogenic effects (World Health Organization, 2020).

Sperm DNA fragmentation (SDF) has been associated with male infertility (Panner Selvam *et al*., 2020). Typically, the harm caused by reactive oxygen species (ROS) leads to the breaking apart of DNA. Direct or indirect damage from ROS leads to aberrant apoptosis and the occurrence of single- or double-strand breakage. Sperm DNA fragmentation can be caused by both internal factors, such as leukocytes, insufficient germ cell maturation, oxidative stress, and incomplete apoptosis, as well as external factors, such as smoking, heat exposure, radiation, chemotherapy, and environmental pollutants (Agarwal *et al*., 2020; Esteves *et al*., 2020). Elevated SDF has been associated with reduced sperm motility, decreased fertilization, and recurrent miscarriages (Alahmar *et al*., 2020). Antioxidants have been used as a treatment for male infertility due to their ability to decrease high levels of ROS, which leads to increased OS. The therapeutic effectiveness of antioxidants in patients undergoing assisted reproduction has been largely overlooked in research. While a generally favorable outcome has been noted, the optimal antioxidant treatment plan and specific therapeutic application have yet to be determined. Infertility is an increasingly significant medical issue due to the declining reproductive capacity of couples worldwide (Palani *et al*., 2020).

The main aim of this interventional clinical research study is to assess the effect of oral antioxidant supplements on infertile men and smoker-infertile men who need intracytoplasmic sperm injection (ICSI) as a treatment method.

MATERIALS AND METHODS

Every case was gathered from Al-Azhar University in Cairo, Egypt's International Islamic Centre for Population Studies and Research (IICPSR)-ART section. Informed permission was acquired from every participant. A formal Ethical clearance letter was obtained from Fayoum University Supreme Committee for Scientific Research Ethics (FU-SCSRE). Code number of the proposal: (EC 23200).

Inclusion Criteria:

A- Female Age ranges between 25-35 years old.

B- No medical diseases male and female (e.g. liver and kidney diseases).

C- Mild male factor (no severe male factor or azoospermia).
D- No previous testicular or scrotal operation.

This Study Will Deal With 100 Infertile Couples (200 samples) Divided Into Two Groups:

Group 1: infertile couples due to male partner's factor (50 couples).
Group 2: infertile couples due to male partner's factor and smoking (50 couples).

Before undergoing ICSI, all male partners were given antioxidant therapy in the form of FERTITONE X Tablets, 1 mg twice a day in the morning and evening for three months. Semen samples were obtained from the patients under investigation both at the start and three months into the trial.

All male partners undergo ICSI taking antioxidants before ICSI.

Samples: Fresh semen sample. In this study, All semen samples were investigated (motility, count and abnormality) before and after treatment with antioxidants and ICSI outcome.

All Males Subjected To:

1-Complete semen analysis (volume, concentration, motility).
2-Sperm DNA Fragmentation Index.
3-Administration of antioxidant treatment for at least two months prior to their partner's ICSI cycle.
4-ICSI outcomes after treatment (fertilization rate, embryo quality, pregnancy rate)
5-Analyzing the data statistically and writing the results obtained.

The Male Patients Were Subjected To:

1-History: Every patient's complete medical history was obtained, including information on age, length of marriage, employment, smoking, drug use, exposure to heat, chemicals, or radiation, surgeries, and past medical histories of chronic illnesses.

2-Complete Semen Analysis:

i. Sample Collection:

After two or five days of abstinence, semen samples are obtained by masturbation; longer abstinence intervals (10 days) result in reduced motility, while shorter periods provide low volume and density (WHO, 2020). To reduce collection mistakes, the container needs to be sterile, clean and wide-mouthed, and it should be from a batch that has been shown to be non-toxic to spermatozoa. Within an hour of collection, the semen samples should be evaluated and kept at room temperature or body temperature (WHO, 2020).

ii. Physical Examination:

A)-Liquefaction: At room temperature, a typical semen sample liquefies in 60 minutes, but it frequently does so in 15 minutes. It should be noted that total liquefaction takes more than sixty minutes to happen. Gelatinous entities, or grains that resemble jelly, can be seen in normal semen samples. These grains do not liquefy and do not seem to have any clinical importance (WHO, 2020).

B)-Semen Volume: is by filling a sterile, heated graduated glass pipette with the entire ejaculate.

C)-Semen pH: A pH paper ranging from 6.0 to 10.0 was used to estimate the pH of semen. It is best to measure the pH at a consistent time after liquefaction, ideally half an hour after ejaculation, but at least an hour later.

iii.Microscopic Examination:

Include analysis of the motility, morphology, and concentration of sperm.

A) Sperm Concentration:

A sterile glass slide with a fixed volume of 10 m of semen is pipetted into it and covered with a cover slip measuring 22 by 22 mm. Spreading the sample for easy sight is the weight of the coverslip. A bubble between the cover slip and the slide should not form or become trapped. One sperm in a microscopic field at 400X equals approximately one

million/ml of seminal fluid.

B) Sperm Motility:

A drop of well-mixed undiluted sperm is placed on the surface of a warm, dry, and clean microscopic slide, followed by a cover slip. Allow the slide to rest on the bench or on the microscopic stage until the fluid movement stops. The sperm drop is then examined under a 400 X magnification microscope, preferably with phase contrast. At least five distinct microscopic fields are used to count both motile and immotile sperm, and at least 200 spermatozoa should be assessed. The mean value is used to calculate the percentage motility.

C) Categories of Sperm Movement:

1-Progressive motility (PR): spermatozoa moving actively.

2-Non-progressive Motility (NP): all other patterns of motility with an absence of progression.

3-Immotility (IM): no movement

D) Sperm Morphology:

The examination of sperm morphology using rigorous criteria involves a comprehensive process that starts with the preparation of clean microscope slides and continues with the proper fabrication of thin semen smears and slide evaluation.

Sperm Preparation Technique:

All males were subjected to sperm preparation before ICSI:

1. Mix the sample of semen thoroughly.
2. To encourage the clearance of seminal plasma, dilute the total semen sample 1 + 1 (1:2) with the supplemented medium.
3. Divide the diluted mixture among many centrifuge tubes, ideally using no more than three milliliters in each tube.
4. Centrifuge for five to ten minutes at 300–500 g.
5. Gently remove the supernatants by aspirating them carefully.
6. Gently pipette the mixed sperm pellets back into suspension in 1 milliliter of enriched media.
7. Centrifuge once more for 3-5 minutes at 300–500g.
8. Gently aspirate the supernatant and dispose of it.
9. Gently pipette the sperm pellet back into suspension in a volume of enriched media that is suitable for disposal at the end.

Sperm DNA Fragmentation Index:

The method of sperm DNA fragmentation index is TUNEL assay. The TUNEL evaluation was carried out using an in situ cell death detection kit. For 30 seconds, the air-dried smeared sample was fixed in 3.9% paraformaldehyde at 28°C and further washed with phosphatebuffered saline (PBS) at a pH of 7.4 and then permeabilized with 2% Triton X-100. Under sterile conditions, the nucleotide mixtures labelled with TdT were layered onto individual slides and incubated in a humidified chamber at 37°C for 58 minutes in the absence of light. Subsequently, the humidified slides were washed three times and stained with 8 mg/mL diamidino-2-phenylindole (DAPI), and negative controls without the TdT-tagged enzyme were run in each duplicate for each sample. A total of 300 sperm per entity were examined using fluorescence microscopy by the same surveyor. The spermatozoa stained with DAPI (blue) were counted first, followed by the spermatozoa dyed green (TUNEL-positive), and then the percentage of these cells in the total sample was calculated.

All Females Subjected to:

Controlled Ovarian Hyperstimulation: All the female partners underwent ovulation induction using a short or long protocol. Oocytes-cumulus complexes (OCC) were recovered 36 h after the administration of 5000 or 10000 IU of human chronic

gonadotrophins (HCG).

Collection of Human Cumulus Cells: Oocytes were aspirated under general anesthesia under transvaginal ultrasonography, approximately 34-36 hours after the HCG administration. Follicular fluid was aspirated into sterile tubes 14 ml. The Oocyte-cumulus cells (OCC) complexes were isolated under a dissecting microscope and washed then the corona cells were removed.

Intracytoplasmic Sperm Injection (ICSI): Intra cytoplasmic sperm injection was performed according to the protocol of Van Steirteghem. Each oocyte was injected with a single morphologically normal and immobilized polyvinyl Pyrolidone (PVP) spermatozoon.

Assessment of Fertilization: Assessment of fertilization and embryo development: Fertilization will be confirmed after 16–18 h by the observation of two distinct pronuclei (2PN) and two polar bodies.

Embryo Transfer and Pregnancy Assessment: Embryo transfers will be performed on day 2 or 3 according to the number and quality of the embryos. B-HCG levels in the blood will be measured to detect a positive pregnancy. B-HCG assays were achieved by electrochemiluminescence immunoassay (Roche-Hitachi Cobas e 411) using quantitative kits (Cobas, Japan).

Statistical Analysis:

We compared ICSI cycle parameters between the first and second trials in each group. The statistical and data analysis for this study involved several key steps. Initially, continuous variables such as sperm count, motility, and morphology were analyzed using measures such as means, standard deviations, and ranges.

For inferential statistics, comparisons between pre-and post-treatment semen parameters within each experimental group (Group 1 and Group 2) were conducted using paired t-tests or non-parametric equivalents if assumptions were not met. Differences in fertilization rates between groups were assessed using chi-square tests or Fisher's exact tests where appropriate. Additionally, logistic regression models may have been employed to evaluate the association between antioxidant treatment and clinical pregnancy rates, adjusting for potential confounding variables.

Statistical significance was typically set at $p < 0.05$. Data analysis was performed using statistical software such as SPSS or R, ensuring rigorous evaluation of treatment effects and outcomes related to intracytoplasmic sperm injection (ICSI) success and pregnancy rates.

RESULTS

Semen Analysis:

Our result in Table 1, revealed that there was a significant change in sperm concentration ($P = 0.0484$), liquefaction time ($P = 0.1157$), PH of semen ($P = 0.6269$), and semen volume ($P = 0.1434$) at the start of the trial and after three months of treatment, with variations that were not statistically significant. Nevertheless, following three months of treatment, there were statistically significant differences ($P < 0.0001$) in the rise in both sperm motility and progressive motility.

Table 1: Comparison between semen parameters of the studied group 1 at the beginning study versus after 3 months of treatment (n=50):

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
Volume /ml	2.25 ± 0.30	2.34 ± 0.31	1.475	0.1434
Liquefaction time/min	16.07 ± 3.20	15.07 ± 3.10	1.587	0.1157
PH of semen	7.13 ± 0.21	7.11 ± 0.20	0.488	0.6269
Concentration (mill/ml)	16.05 ± 2.20	17.15 ± 3.21	1.999	0.0484
Sperm Motility (%)	42.71 ± 4.10	58.61 ± 3.90	19.86	**< 0.0001**
Progressive Motility (%)	11.21 ± 4.62	29.21 ± 4.32	20.12	**< 0.0001**

Table 2, showed a low change in semen volume (p= 0.7648) and PH of semen (p= 0.1855), at the beginning of the study and after 3 months of treatment. Furthermore, there was a significant (P < 0.0001) decline in the liquefaction time. However, there was an increase in sperm concentration, sperm motility, and progressive sperm motility which improved after 3 months of treatment with statistically significant differences (P < 0. 0001).

Table 2: Comparison between semen parameters of the studied group 2 at the beginning study and after 3 months of treatment (n=50):

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
Volume /ml	2.31 ± 0.50	2.34 ± 0.50	0.300	0.7648
Liquefaction time/min	18.01 ± 2.00	15.07 ± 2.00	7.350	**< 0.0001**
PH of semen	7.29 ± 0.30	7.21 ± 0.30	1.333	0.1855
Concentration (mill/ml)	17.12 ± 2.20	19.15 ± 2.21	4.603	**< 0.0001**
Sperm Motility (%)	40.51 ± 3.10	59.93 ± 3.21	30.772	**< 0.0001**
Progressive Motility (%)	13.11 ± 5.01	29.21 ± 5.02	16.052	**< 0.0001**

Sperm morphology:

After 3 months of treatment, Table (3), demonstrated a statistically significant (P<0.0001) decrease in aberrant forms, sperm head defects, sperm mid-piece defects, and sperm tail abnormalities in the examined group 1.

Table 3: Comparison between sperm morphology of the studied group 1 at the beginning study and after 3 months of treatment (n=50).

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
Abnormal forms	96.4 ± 1.2	95.1 ± 0.2	7.556	P<0.0001
Sperm Head defects	80.4 ± 2.8	78.0 ± 1.5	5.343	P<0.0001
Sperm midpiece defects	40.3 ± 2.9	35.0 ± 0.9	12.342	P<0.0001
Sperm Tail defects	20.6 ± 2.4	18.2 ± 2.0	5.432	P<0.0001

The data obtained in Table (4), showed a decrease in abnormal forms, sperm Head defects, sperm mid-piece defects, and sperm tail defects in group 2 at the beginning study and after 3 months of treatment with statistically significant differences (P 0. 0001).

Table 4: Comparison between sperm morphology of the studied group 2 at the beginning study and after 3 months of treatment (n=50).

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
Abnormal forms	**97.4 ± 1.1**	**96.2 ± 1.2**	**5.212**	**< 0.0001**
Sperm Head defects	**83.4 ± 1.8**	**80.9 ± 1.6**	**7.340**	**< 0.0001**
Sperm midpiece defects	**30.3 ± 2.0**	**25.0 ± 2.0**	**13.250**	**< 0.0001**
Sperm Tail defects	**28.6 ± 2.0**	**20.9 ± 1.9**	**19.737**	**< 0.0001**

Sperm DNA Fragmentation Index (DFI)

Table 5, clarifies that, after three months of therapy, the levels of the sperm DNA fragmentation index in the investigated group 1 were significantly (P<0.0001) lower than the index's value at the start of the study.

Table 5: Comparison between DFI of the studied group 1 at the beginning of the study versus 3 months of treatment (n=50):

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
DNA fragmentation index	25.20 ± 3.0	22.00 ± 3.1	5.245	**P<0.0001**

Table (6) , showed a highly significant decrease (P < 0. 0001) in sperm DNA fragmentation index in the studied group 3 (infertile smoker men) after 3 months of treatment with FERTITONE X in comparison to the beginning of the study.

Table 6: Comparison between sperm DFI of the studied group 2 at the beginning study and after 3 months of treatment (n=50):

Parameters	Initial	After treatment	t-statistic	P value
	Mean ± SD	Mean ± SD		
DNA fragmentation index	30.30 ± 3.0	26.00 ± 2.1	8.303	**P<0.0001**

ICSI Outcome:

The results obtained from ICSI outcomes in females are given in Table (7). The data showed a decrease in the total number of oocytes collected number in the ICSI trial after treatment compared to the previous ICSI trial before treatment with statistically

insignificant differences. (P =1.000). However, there was an increase in mature oocytes number, fertilization rate on Day 1, and cleavage rate on Day 3 in the second ICSI trial after treatment in comparison to the previous ICSI trial (0.9611, 0.0233, and 0.1288 respectively). The incidence of embryo development on Day 5 showed a marked increase in blastocyst formation rate in the second ICSI trial after treatment compared to the previous ICSI trial with statistically significant differences (P < 0.001).

Table 7: ICSI outcome in female studied group 1 regarding the previous ICSI trial versus the second ICSI trial treatment:

Parameters	First ICSI trial Before n= 459	Second ICSI trial After treatment n= 411	Difference %	P value
	Mean (%)	Mean (%)		
Total Collected number	459 (100.0%)	411 (100.0%)	00 %	1.000
Mature oocytes number	323 (70.37%)	290 (70.55%)	0.18 %	0.9611
Fertilization rate on Day 1	225 (69.65%)	229 (78.96%)	9.31%	0.0233
Cleavage rate on Day 3	213 (65.94%)	211 (72.75%)	6.81%	0.1288
Blastocyst rate on Day 5	138 (42.72%)	183 (63.10%)	20.38%	<0.001

Table (8), showed a decrease in the total number of oocytes collected number in the second ICSI trial after treatment compared to the previous ICSI trial with statistically insignificant differences (P = 1.000). However, there was a decrease in mature oocytes number in the second ICSI trial after treatment compared with the previous ICSI trial with statistically insignificant differences (P = 0.728). On the contrary, there was a noticeable increase in fertilization rate on Day 1, cleavage rate on Day 3 and blastocyst formation rate on Day 5 in the second ICSI trial after treatment in comparison to the previous ICSI trial with statistically significant differences (P < 0.001).

Table 8: ICSI outcome in female studied group 2 regarding the previous ICSI trial versus the second ICSI trial after treatment:

Parameters	First ICSI trial Before	Second ICSI trial After treatment	Difference %	P value
	Mean (%)	Mean (%)		
Total Collected number	499 (100.0%)	490 (100.0%)	00 %	1.000
Mature oocytes number	349 (69.9%)	348 (71.1%)	1.2 %	0.728
Fertilization rate on Day 1	219 (62.7%)	265 (76.2%)	13.5%	< 0.001
Cleavage rate on Day 3	209 (59.8%)	249 (71.5%)	11.7 %	0.001
Blastocyst rate on Day 5	159 (45.5%)	201 (57.7%)	12.2%	0.001

Pregnancy Rate:

In the second ICSI experiment following therapy, the pregnancy rate among the female patients in all groups (Group 1 and Group 2) increased noticeably (Table 9). Nonetheless, the Second ICSI experiment showed an increase in the pregnancy rate. There were no statistically significant changes between the post-treatment data and the previous ICSI experiment in any of the female investigated groups (P = 0.684, 0.318, and 0.058, respectively).

Table 9: Pregnancy rate among female studied patients regarding theprevious ICSI trial vs the second ICSI after treatment:

+ve Pregnancy	First ICSI trial Before n= 50	Second ICSI trial After treatment n=50	Difference %	P value
	Mean (%)	Mean (%)		
Group 1	19 (38.0%)	21 (42.0%)	4%	0.684
Group 2	20 (40.0%)	29 (59.0%)	19%	0.058

DISCUSSION

Our research showed that the antioxidant therapy (AOX therapy) improved the clinical pregnancy rate, semen motility, sperm abnormalities and OS levels in men who underwent a thorough diagnostic evaluation that excluded major concurrent conditions, genetic, anatomical, inflammatory, traumatic, or testicular causes of male infertility, as well as associated female infertility.

Majzoub and Agarwal (2018) discovered a positive association between increased levels of sperm concentration, motility, and morphology, and the consumption of several nutrients including vitamin E, vitamin C, N-acetylcysteine (NAC), carnitines, Coenzyme Q10 (CoQ10), lycopene, selenium, and zinc, as revealed in a previous investigation involving 21 participants. Similar to our study, we used FERTITONE X tablets which contain L-Carnitine, L-Arginine, Coenzyme Q10, Selenium, Zinc, Folic acid and Vitamins (C, E, B6, B12 & D3). This study's findings suggested that there was negligible fluctuation in sperm concentration, and semen pH when comparing the initial and three-month periods of antioxidant treatment. After receiving a three-month antioxidant treatment, there was a noticeable increase in both sperm motility and progressive motility.
Antioxidants (e.g., vitamin C, vitamin E, glutathione, albumin, carotenoids, or uric acid) were plentiful in the seminal plasma. They act to avoid sperm fragmentation caused by ROS following ejaculation. When OS occurs, these antioxidants would be inadequate. An imbalance of free radicals and antioxidant levels leads to an OS event which reduces sperm quality. Therefore, sufficient antioxidants should be available to resist the abundance level of free radicals such as ROS (Di Meo & Venditti, 2020). Antioxidant scavenging structures have a critical part to play in the process of inactivating ROS. Various antioxidant supplements and combinations of regimens, such as vitamins C and E, selenium, zinc, and glutathione, have long been used as treatments for male infertility. The correlation between SDF and semen ROS is the fundamental value of antioxidant consumption. It is purposed to improve sperm quality (Martinez *et al*., 2020).

Scaruffi *et al*. (2021) evaluated reproductive outcomes of IVF cycles after treatment with 2 Gametogen® tablets that contained myo-inositol (1000 mg), alpha-lipoic acid (800 mg), folic acid (400 mg), coenzyme Q10 (200 mg), zinc (15 mg), and selenium (83 µg) and vitamins B2 (2.8 mg), B6 (2.8 mg), and B12 (5 µg). The study exhibited significant progressive sperm motility and pregnancy rate ($P < .001$). Pregnancy rate increased from 3% pre-treatment to 33% 12 weeks post-treatment.

In the study conducted by Smits *et al*. (2019), the researchers proposed the hypothesis that zinc (Zn) supplementation may increase sperm concentration. Zn plays a role in epithelial integrity, showing that Zn is essential for maintaining the lining of the reproductive organs. Furthermore, it has an important role in stabilizing the cell membrane and nuclear chromatin of spermatozoa in seminal plasma. It may have a regulative role in the progress of capacitation and acrosome reaction. The Zn concentration in human seminal plasma is higher than in other tissues. Zn has been shown to be vital for

spermatogenesis. It plays a significant role in testis development and sperm physiologic functions (Vickram *et al*., 2021). Zn contains a variety of roles in the spermatogenesis phases. For example, in the initiation of spermatogenesis, Zn is important in the participation of ribonuclease activities that are highly active during the mitosis of spermatogonia and meiosis of spermatocytes. At the end of spermatogenesis, Zn is highly concentrated in the tail of mature spermatozoa and involved in sperm motility (Fallah *et al*., 2018).

Many studies have shown that Zn has antioxidative activities and has a main role in scavenging ROS. ROS (*e.g*.: superoxide [O_2^-] anion, hydrogen peroxide [H_2O_2], peroxyl [ROO^-] radical and the very reactive hydroxyl [OH^-] radical) are unstable compounds with a short half-life that can adversely influence certain cellular functions. High ROS levels affect sperm function by oxidation of lipids, proteins, and DNA (Pisoschi *et al*., 2021). Increased ROS levels in the seminal plasma of infertile men may decrease Zn concentration, increasing the detrimental effects of ROS on sperm cells that are correlated with abnormal sperm parameters. Several studies suggested the hypothesis that decreased Zn concentration can lead to an increase in the oxidation of DNA, proteins and lipids which underlined Zn main role in the prevention of oxidative damage. Zn is present at high concentrations in the seminal fluid and there is evidence that Zn appears to be a potent scavenger of excessive generation of superoxide anions produced by abnormal spermatozoa and/or leukocytes in human semen after ejaculation. The abnormal spermatozoa would be a source of superoxide anions that bind with Zn present in the seminal plasma and thus decrease the Zn levels (Aitken & Drevet, 2020).

Anakwe *et al*., (2022) proposed that individuals who are trying to conceive should either completely refrain from smoking or decrease their smoking habits. Nevertheless, it is crucial to acknowledge that the existing research on the impact of smoking on sperm characteristics, such as concentration, motility, and morphology, is of restricted quality due to a substantial probability of bias. A statistically significant decrease ($P<0.0001$) in the occurrence of abnormal forms of sperm, including abnormalities in the sperm head, mid-piece, and tail, was observed after a three-month intervention in a group of male individuals with infertility who smoke, referred to as group 2. Moreover, there was a significant increase in both the concentration and motility of sperm, including progressive motility. This observation aligns with a study conducted by Bundhun *et al*. (2019) that investigated the influence of smoking on sperm quality by comparing semen characteristics between smokers and non-smokers. Smoking was found to have a harmful effect on the shape and structure of sperm cells, resulting in a higher occurrence of abnormalities. Furthermore, clinical outcomes have demonstrated that smoking has an adverse impact on sperm concentration.

Zhang *et al*. (2023) showed that cigarette smoking influences both Ca2+-ATPase activity and the motility of spermatozoa, by increased seminal cadmium and Zn concentrations reduction. Therefore, it has been indicated that cigarette smoking can increase oxidative damage via decreasing seminal plasma Zn concentration and other antioxidants, thereby affecting sperm parameters. Bazid *et al*. (2022) illustrated a negative relationship between cigarette smoking and seminal plasma Zn concentration. Heavy smoking was associated with low sperm count, low motility, poor morphology and increased seminal cadmium levels so Zn therapy improved sperm quality.

Studies by Busetto *et al*. (2018) used Proxeed Plus supplements that consisted of 1000 mg l-carnitine, 725 mg fumarate, 500 mg acetyl-l-carnitine, 1000 mg fructose, 20 mg coenzyme Q10, 90 mg vitamin C, 10 mg zinc, 200 μg folic acid, and 1.5 μg vitamin B12 for 6 months. They showed an increase in sperm motility and morphology and a decrease in SDF that were statistically significant. l-Carnitine and acetyl-l-carnitine are known to play a key part in spermatozoa energy metabolism. Clinical studies have previously

revealed that oral administration of these compounds to asthenozoospermic individuals increases the percentage of total sperm motility, progressive sperm motility, average speed, and linearity of sperm motility. Noegroho *et al*. (2022) administered a combination of vitamins, and antioxidants consisting of l-carnitine 750 mg, coenzyme Q10 10 mg, and folic acid 100 µg or mcg (microgram), and oligo-elements (zinc 5 mg and selenium 25 mg) which also showed similar results as aforementioned studies except for an increase of total sperm motility. Pregnancies were also reported in this study in 3 patients who were given antioxidants and in 4 patients in the placebo group. The association between pregnancy and DFI levels is known. Low DFI levels (<30%) can decrease the spontaneous pregnancy occurrence. The results of our study demonstrated a substantial reduction ($P<0.0001$) in the sperm DNA fragmentation index among the subjects in group 2, which comprised infertile male smokers, following a three-month course of treatment with FERTITONE X. This decrease was observed relative to the initial measurements taken at the beginning of the study.

Aboulmaouahib *et al*. (2017) suggested that smoking cigarettes has the potential to decrease the SDF value. Multiple studies have produced inconclusive findings regarding the influence of smoking on SDF, some studies have found no noticeable impact, and others have suggested that smoking has a harmful influence on SDF. According to an alternative study, it has been noted that smoking negatively impacts the value of sperm DNA fragmentation (SDF) in men who are facing infertility. The observed phenomenon exhibited a greater degree of prominence among individuals who were heavy smokers, as opposed to those who were moderate or nonsmokers (Salonia *et al*., 2019). Infertile men were used in several of the included experiments, after which they were administered AOX. The current study enhanced the ICSI outcome compared to the previous review. This allowed us to increase the level of evidence for many of the outcomes under investigation and validate the findings of the Cochrane analysis (Smits *et al*., 2019).

The number of mature oocytes, the rate of fertilization on Day 1, and the rate of cleavage on Day 3 in the second ICSI trial after therapy were all higher than in the first. Furthermore, compared to the previous ICSI study, a significant increase in the blastocyst formation rate was noted in the incidence of embryo development on Day 5. However, a multicenter, double-blind, randomized, placebo-controlled trial conducted in the United States between 2015 and 2018 and published in 2020 found that an antioxidant formulation containing vitamins C, E, selenium, L-Carnitine, zinc, folic acid, and lycopene had no effect on sperm quality parameters, pregnancy, or live birth rates (Steiner *et al*., 2020)[31]. The results of the current investigation demonstrated that the second ICSI trial after treatment revealed an increase in fertilization rate on Day 1, cleavage rate on Day 3, and blastocyst formation rate on Day 5, in comparison to the first ICSI study carried out prior to therapy.

Treating infertile males with AOXs has two key goals: decreasing the rate of miscarriages and increasing the pregnancy rate. The results showed that after treatment, the pregnancy rates in all three of the female study groups (Group 1 and Group 2) significantly increased. In the Second ICSI study, there was a rise in the pregnancy rate. Between the previously conducted ICSI trial and the all-female examined group, there were no statistically significant alterations following treatment. These results are consistent with the most recent Cochrane review and meta-analysis conducted by Smits *et al*. (2019). According to a number of reviews, AOX treatment is effective for clinical pregnancy following aided or spontaneous reproduction (Majzoub and Agarwal, 2018).

However, the MOXI trial did not find any evidence that treating AOX would improve clinical pregnancy rates in infertile males; instead, the same clinical pregnancy rates were observed when treating AOX (Steiner *et al*., 2020).

Similarly, total antioxidant capacity (U/mL) was correlated with a greater pregnancy rate in both the study and control groups. This implies that during the oocyte maturation phase, both groups responded well to antioxidants, resulting in mature oocytes and superior embryos that were prepared for implantation (Zaha *et al*., 2023).

From conception to pregnancy, ROS is crucial to a woman's reproductive process. Pregnancy success appears to be substantially better predicted by the ratio of pro-oxidants to antioxidants than by ROS or antioxidant levels alone. This ratio represents the entire oxidative stress environment. Recent studies suggest that the secret to developing high-quality embryos that lead to better pregnancy rates during IVF cycles may lie in antioxidant supplements, precision embryo culture, and safe laboratory conditions (Mauchart *et al*., 2023).

CONCLUSION

The FERTITONE X drug has a clear anti-oxidant effect on infertile men, resulting in decreased liquefaction time and increased volume, concentration, sperm motility, and progressive motility. It also improved the index of abnormal forms and sperm DNA fragmentation. Furthermore, FERTITONE X treatment improved the ICSI outcome and pregnancy rate in the Second ICSI trial.

Declarations:

Ethical Approval: The study was approved by the Fayoum University Supreme Committee for Scientific Research Ethics (FU-SCSRE). Code number of the proposal: (EC 23200).

Conflict of interests: The authors declare no conflicts of interest.

Authors Contributions: All authors contributed equally, and have read and agreed to the published version of the manuscript.

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