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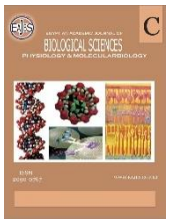

The Effect of Oral Antioxidant Supplementation on Infertile Male, Infertile Obese Male, Intracytoplasmic Sperm Injection (ICSI) Outcome and Pregnancy Rate

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ABSTRACT

Background: Oxidative stress, caused by an imbalance between reactive oxygen species and antioxidants in semen, can harm sperm deoxyribonucleic acid and lipids, leading to lower success rates in intracytoplasmic sperm injection. Recent research suggests that oral antioxidant supplements may alleviate this stress and improve sperm quality. However, the evidence is limited. Thus, this study aims to assess the effectiveness of these supplements in enhancing intracytoplasmic sperm injection success rates, potentially providing a solution for male.**Methods:** This study involved two groups of 100 infertile couples. Group 1: infertile couples due to male partner's factor. Group 2: infertile couples due to male partner's factor and obesity. All male partners received antioxidant therapy with FERTITONE X pills, taking 1 mg twice daily, once in the morning and once in the evening, for three months. Semen samples were collected from participants before and after treatment. Additionally, the outcomes of intracytoplasmic sperm injection and pregnancy rates were evaluated.**Results:** The major finding from this study was that antioxidant therapy led to significant improvements in fertilization ($p < 0.001$) and cleavage ($p < 0.001$) in group 2, along with a notable increase in clinical pregnancy rates (42.0% to 52.0%). Significant changes in routine sperm parameters were also observed in all groups. **Conclusion:** In conclusion, antioxidant therapy with FERTITONE X pills demonstrated substantial benefits in improving fertilization and cleavage rates among infertile couples undergoing intracytoplasmic sperm injection. The observed increase in clinical pregnancy rates further supports the potential efficacy of antioxidant supplementation in enhancing reproductive outcomes in male infertility treatments. These findings underscore the importance of antioxidant management in improving sperm quality and subsequent fertility outcomes.

INTRODUCTION

Infertility remains a major global and public health challenge, affecting people of all genders in human society. The fifth most significant disability in the world, infertility has a detrimental effect on the self-esteem of individuals who experience it. Women continue to bear a greater social burden from these adverse side effects than men (Zayed and El-Hadidy, 2020).

According to Harrison *et al.*(2021), the most common causes of male infertility include defects in sperm motility and morphology, low or absent sperm counts, and changes in semen ejection. On the other hand, abuse of alcohol intake, smoking, exposure to environmental toxins like heavy metals or pesticides, excessive heat, psychological stress, prolonged intense physical activity, high-fat and high-protein diets, drug-induced stress (like marijuana), anabolic steroid intake, obesity, aging, and stress brought on by reproductive tract infections are just a few of the factors that can cause the production of reactive oxygen species (ROS) (Sengupta *et al*., 2020). Over the past 25 years, there has been a definite link between obesity and poor reproductive results; however, the underlying processes are still unknown (Zhu *et al*., 2022). Obesity in males has been shown to have detrimental effects on sperm qualities including count, motility, and morphology in addition to fertility, despite the fact that obesity in females is well known to have harmful effects on pregnancy and the health of the fetus (Venigalla *et al*., 2023). ROS-mediated damage frequently results in DNA fragmentation. Damage caused by ROS, either direct or indirect, causes aberrant apoptosis and single- or double-strand breaks. Sperm DNA fragmentation can result from both intrinsic and extrinsic causes, including inadequate germ cell development, Oxidative stress (OS), smoking, heat exposure, chemotherapy, and environmental pollutants. ROS and antioxidants have been demonstrated to be negatively correlated; infertile men can produce excessive amounts of ROS due to inadequate seminal antioxidant potential (Agarwal and Sengupta, 2020).

Furthermore, because of their capacity to counteract elevated ROS levels that result in a high OS, antioxidants have long been utilized to treat male infertility. The clinical efficacy of antioxidants in patients receiving assisted reproduction has not received much attention in research. Although a generally positive result has been observed, the ideal antioxidant regimen and particular clinical indication are still unknown. Due to a decline in couples' ability to reproduce globally, medical concern over infertility has increased recently (Palani *et al*., 2020).

Assisted reproductive techniques (ARTs) are a medical treatment option that has gained popularity among infertile couples. Between 2012 and 2016, ART utilization grew at varying rates throughout Europe, the USA, New Zealand, and Australia (De Geyter *et al*., 2020). Throughout these territories, 1.3 million ART cycles were completed in 2016. Numerous causes, including socioeconomic and environmental ones, could be the source of this increase (Skakkebæk *et al*., 2021). This interventional clinical research study aims to assess the effect of oral antioxidant supplements on the outcome of intracytoplasmic sperm injection (ICSI) in infertile patients who need ICSI to treat their infertility.

Research into oral antioxidant supplements for improving ICSI success reveals critical gaps. Firstly, the absence of long-term studies limits understanding beyond immediate outcomes. Secondly, diverse dosage and duration protocols hinder the establishment of standardized guidelines, crucial for optimizing treatment efficacy. Thirdly, while antioxidants improve sperm quality by reducing oxidative stress, their precise mechanistic pathways during ICSI are poorly understood. Further research into these mechanisms is needed to refine therapeutic strategies and enhance treatment precision in male infertility management.

Moreover, current studies frequently concentrate on specific demographic groups, curtailing the generalizability of outcomes. Inclusive research across diverse populations is imperative to establish the broader applicability and effectiveness of antioxidant interventions in enhancing ICSI success rates. Additionally, there is a shortage of research investigating the potential synergies of combining antioxidant supplementation with other therapeutic interventions during ICSI.

Exploring these combined approaches may unveil novel strategies to enhance reproductive outcomes in male infertility cases.

MATERIALS AND METHODS

All 100 infertile couples in this study were provided by the International Islamic Centre for Population Studies and Research (IICPSR)-ART section at Al-Azhar University in Cairo, Egypt. Each participant gave their consent after being fully informed. There are two groups undergoing ICSI and all males of the two groups take antioxidant treatment. Informed permission was acquired from every participant. A formal Ethical clearance letter was obtained from the Fayoum University Supreme Committee for Scientific Research Ethics (FU-SCSRE). Code number of the proposal: (EC 23200).

Experimental Groups:

Group 1: infertile couples due to male partner's factor (50 couples) before and after the oxidant treatment.

Group 2: infertile couples due to male partner's factor and obesity (50 couples) before and after the oxidant treatment.

All male partners in Group 1 and Group 2 undergo ICSI and were treated with antioxidant therapy before ICSI in the form of FERTITONE X tablet 1 mg twice a day morning and evening for three months. The semen samples were collected from the studied patients at the beginning and after three months of the study. Duration of the treatment should be adjusted according to several factors including the detected levels of ROS and antioxidant enzymes in seminal fluid and spermatozoa. The duration of an antioxidant therapy would have to be adjusted according to the place where the damage occurs. If it is in the epididymis, a treatment course of at least two weeks should be sufficient to counteract ROS-induced damage. In addition, since oxidative stress in the epididymis is a constitutive process and antioxidants have no side effects, antioxidant therapy should be recommended until pregnancy is achieved. This would apply to couples undergoing timed intercourse as well as to couples undergoing *in vitro* fertilization. On the other hand, if the oxidative damage is occurring in the testes such as in the case of clinical varicocele, the duration of antioxidant treatment should be at least three months. We take Semen analysis before and after treatment from all males in two groups. Also, Female treatment of the experimental groups included more than one stage. Firstly, Controlled Ovarian Hyper Stimulation: Using a short or long protocol, ovulation induction was performed on the partners of all females. Oocytes-cumulus complexes (OCC) were recovered 36 hours after 5000 or 10000 IU of human chronic gonadotrophins (HCG) were administered. Secondly, Collection of Human Cumulus Cells: The HCG injection after 34–36 hours, oocytes were aspirated while under general anesthesia and transvaginal ultrasound imaging. Fourteen ml sterile tubes were aspirated of follicular fluid. After separating and cleaning the Oocyte-cumulus cell (OCC) complexes under a dissecting microscope, the corona cells were removed. Third, ICSI: One morphologically normal spermatozoon that had been immobilized in polyvinylpyrrolidone (PVP) was inserted into each oocyte. Evaluation of fertilization: Evaluation of the development of the embryo and fertilization: Fertilization will be evident after 16–18 hours if there are two distinct pronuclei (2PN) and two polar bodies. Finally, Embryo Transfer and Pregnancy Assessment: Day 3 will be used for embryo transfers, depending on the quantity and caliber of the embryos. The blood's B-hCG level will be assessed to determine whether a pregnancy is confirmed.

Statistical Analysis:

We compared ICSI cycle parameters between 2 groups. The statistical and data analysis for this study involved several key steps. Initially, descriptive statistics were employed to summarize the demographic characteristics of the study participants, including age, BMI, and baseline fertility parameters. 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 $p < 0.001$. Data analysis was performed using statistical software such as SPSS or R, ensuring rigorous evaluation of treatment effects and outcomes related to ICSI success and pregnancy rates.

RESULTS

Patients Characteristics:

In this study, all semen samples were investigated (motility, count and abnormality) before and after treatment with antioxidants. ICSI outcomes after treatment (fertilization rate, embryo quality, pregnancy rate) are recorded. The couples in group 1 do not suffer from obesity in contrast to group 2 who suffer from obesity.

From a total of 100 infertile couples undergoing ICSI in this study, only 47 females were pregnant. Clinical pregnancy rates in group 1 in the first and second ICSI were 38.0% and 42.0% respectively. Males are aged from 25 – 35 years. The mean male age is 30. Most of the body mass index (BMI) are from 20 -25 with an average of 22.0. The infertility years are from 3 to 5 years and all couples undergo two ICSI trials Table (1).

Table 1: Characteristics of the male studied group 1 in the previous trial vs, the second trial after treatment (n=50):

Parameters	First ICSI trial	Second ICSI trial	t-statistic	P value
	Mean ± SD	Mean ± SD		
Male Age	30.01 ± 2.11	30.41 ± 2.11	0.948	0.3455
Male BMI (Kg/m^2)	22.20 ± 1.00	22.20 ± 1.00	0.000	1.000
Infertility years	4.61 ± 2.0	4.92 ± 2.0	0.775	0.4402

Semen Analysis:

Our result in Table 2 that there was a minimal change in sperm concentration (1.999), liquefaction time (1.587), pH of semen (0.488), and semen volume (1.475) 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 and progressive motility.

Table 2: Comparison between semen parameters of the studied group 1 (infertile couples due to male partner's factor (50 couples) before and after the oxidant treatment) at the beginning study versus after 3 months of treatment (n=50):

	Parameters	Initial	After treatment	t-statistic	P value
		Mean ± SD	Mean ± SD		
Group 1	**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

As shown in Table 3, there was a low change in semen volume (3.114), pH of semen (1.951), and sperm concentration (1.339), at the beginning of the study and after 3 months of treatment. Also, the data showed a significant decline (5.619) in the liquefaction time. However, sperm motilityand progressive motility increased after 3 months of treatment with statistically significant differences (P □ 0. 0001).

Table 3: Comparison between semen parameters of the studied group 2 (infertile couples due to male partner's factor and obesity (50 couples) before and after the oxidant treatment) at the beginning study and after 3 months of treatment (n=50):

	Parameters	Initial	After treatment	t-statistic	P value
		Mean ± SD	Mean ± SD		
Group 2	**Volume /ml**	2.19 ± 0.31	2.38 ± 0.30	3.114	0.0024
	Liquefaction time/min	15.07 ± 3.10	12.09 ± 2.11	5.619	< 0.001
	PH of semen	7.12 ± 0.21	7.20 ± 0.20	1.951	0.0540
	Concentration (mill/ml)	16.15 ± 3.20	17.15 ± 4.20	1.339	0.1836
	Sperm Motility (%)	31.61 ± 4.17	47.51 ± 4.13	19.156	< 0.0001
	Progressive Motility (%)	11.01 ± 6.62	25.11 ± 5.52	11.567	< 0.0001

Sperm DNA fragmentation index (DFI):

Table 4, 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 4: 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 (5) showed a marked decrease in sperm DNA fragmentation index in the studied group 2 at the beginning study and after 3 months of treatment with statistically significant differences (P < 0. 0001).

Table 5: 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	35.20 ± 2.0	30.00 ± 2.1	12.679	**P< 0.0001**

Hormonal Profile:

Table 6, showing that the studied group 1 that FSH, LH and PRL showed significantly increased in the second ICSI trial compared to the first ICSI.

FSH, LH and PRL levels displayed significantly higher levels in the second ICSI in the group 2 with value (5.5 ± 1.1, 4.2 ± 1.2 and 22.1 ± 4.0 respectively) comparing with the first ICSI (Table 7).

Table 6: Hormonal profile of the studied group 1 in the previous trial versus the second trial after treatment.

Basal hormones levels	First ICSI trial	Second ICSI trial	t-statistic	P value
	Mean ± SD	Mean ± SD		
FSH (IU/L)	5.1 ± 1.0	5.3± 1.1	0.951	0.3438
LH (IU/L)	4.0 ± 1.1	4.1 ± 1.3	0.415	0.6789
PRL (µg/L)	20.4 ± 4.8	21.2 ± 4.7	0.842	0.4018

Table 7: Hormonal profile of the studied group 2 in the previous trial versus the second trial after treatment:

Basal hormones levels	First ICSI trial	Second ICSI trial	t-statistic	P value
	Mean ± SD	Mean ± SD		
FSH (IU/L)	5.3 ± 1.4	5.5 ± 1.1	0.794	0.4289
LH (IU/L)	4.3 ± 0.9	4.2 ± 1.2	0.471	0.6384
PRL (µg/L)	21.4 ± 3.9	22.1 ± 4.0	0.886	0.3778

Outcome of Intracytoplasmic Sperm Injection:

Fertilization Rate and Embryo Development in Group 1 and Group 2:

Table 8, displays the results of the ICSI test for females. The results demonstrated a statistically negligible drop in the total number of oocytes obtained in the second ICSI trial following therapy as compared to the first ICSI experiment. Compared to the first ICSI experiment, the number of mature oocytes, the fertilization rate on Day 1, and the cleavage rate on Day 3 in the second ICSI trial after therapy showed a statistically insignificant increase (Respectively, 0.9611, 0.0233, and 0.1288). When comparing the second ICSI trial following treatment to the first ICSI trial, the incidence of embryo development on Day 5 revealed a considerable rise in the blastocyst formation rate, with statistically significant differences ($P < 0.001$).

Table 8: ICSI outcome in female studied group 1 regarding the previous ICSI trial versus the second ICSI trial after treatment.

	Parameters	First ICSI trial N= 459	Second ICSI trial N= 411	Difference %	P value
		Mean (%)	Mean (%)		
Group 1	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

According to our findings, there were statistically negligible changes between the total number of oocytes collected in the second ICSI trial after therapy and the previous ICSI experiment before treatment. The incidence of ICSI outcome demonstrated that in comparison to the previous ICSI trial prior to treatment, there were high statistically significant differences (P<0.001) in the number of mature oocytes, fertilization rate on Day 1, cleavage rate on Day 3, and blastocyst formation rate on Day 5 in the second ICSI trial following treatment. (Table 9).

Table 9: ICSI outcome in female studied group 2 regarding the previous ICSI trial versus the second ICSI trial after treatment.

	Parameters	First ICSI trial	Second ICSI trial	Difference %	P value
		Mean (%)	Mean (%)		
Group 2	**Total Collected number**	510 (100.0%)	501 (100.0%)	000 %	1.000
	Mature oocytes number	343 (67.25%)	400 (79.85%)	12.60 %	< 0.001
	Fertilization rate on Day1	229 (66.76%)	352 (88.00%)	21.24%	< 0.001
	Cleavage rate on Day 3	213 (62.09%)	328 (81.25%)	19.16 %	< 0.001
	Blastocyst rate on Day 5	138 (40.2%)	256 (64.00%)	23.80%	< 0.001

Embryos grading:

The data obtained in Table 10, showed a high increase in the number of Grade A embryos in the second ICSI trial after treatment compared to the previous ICSI trial with statistically significant differences (P < 0.001). Also, there was a marked decrease in Grade B embryos number, in the second ICSI trial after treatment in comparison to the previous ICSI trial with statistically significant differences (P = 0.006). However, there was a decrease in Grade C embryo numbers in the second ICSI trial after treatment compared to the previous ICSI trial with statistically insignificant differences (P = 0.010).

Table 10: Embryos grading in female studied group 2 regarding the previous ICSI trial vs, the second ICSI after treatment:

Parameters	First ICSI trial N= 159	Second ICSI trial N= 201	Difference %	P value
	Mean (%)	Mean (%)		
Grade A embryos	89.0 (55.9%)	153 (76.1%)	20.2%	< 0.001
Grade B embryos	49.0 (30.8%)	37.0 (18.4%)	12.4%	0.006
Grade C embryos	21.0 (13.3%)	11.0 (5.5%)	7.8 %	0.010

Pregnancy Rate

The percentage of female patients in each of the two groups (Groups 1, and 2) who became pregnant increased significantly in the second ICSI trial after therapy (Table 11). However, the Second ICSI experiment revealed a rise in the number of pregnancies. For all of the female groups under investigation, no statistically significant variations were found. between the pre-treatment data and the results of the prior ICSI trial (4 % and 10 %, respectively).

Table11: Pregnancy rate among female studied patients regarding theprevious ICSI trial vs the second ICSI after treatment:

+ve Pregnancy	First ICSI trial N= 50	Second ICSI trial N=50	Difference %	P value
	Mean (%)	Mean (%)		
Group 1	19 (38.0%)	21 (42.0%)	4%	0.684
Group 2	21 (42.0%)	26 (52.0%)	10%	0.318

DISCUSSION

The findings of our study indicated that the adoption of antioxidant therapy resulted in a notable enhancement in multiple aspects associated with sperm quality, such as concentration, morphology, as well as progressive and total motility. 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.

In this research, we find that administering antioxidants to obese infertile men in group 2 led to decreases in semen volume, pH levels, concentration, liquefaction time, and abnormal forms. The study's findings revealed that there was an improvement in both sperm motility and progressive motility after a three-month period of therapeutic intervention. Similarly, the relationship between sperm quality and body mass index (BMI) continues to be a subject of ongoing discussion and analysis. Multiple studies have confirmed a positive association between increased body mass index (BMI) and different aspects of sperm quality, such as concentration, motility, and morphology (Sun *et al*., 2017; Ma *et al*., 2018). Inconsistent results were documented in additional studies carried out by Ma *et al*. (2018) and Rufus *et al*. (2018).

Several experiments included the utilization of infertile males, who were subsequently administered antioxidants (AOX). The present investigation demonstrated an improvement in the ICSI outcome when compared to the previous Cochrane review conducted by Smits *et al.* (2019).

The second ICSI trial following therapy exhibited elevated levels of mature oocytes, fertilization rate on Day 1, and cleavage rate on Day 3 compared to the first trial. Nonetheless, a clinical study was carried out in the United States from 2015 to 2018, employing a multicenter, double-blind, randomized, and placebo-controlled design. The findings of this study, which were published in 2020 by Steiner *et al*. (2020) revealed that the antioxidant formulation did not exhibit any discernible effects on the assessed sperm quality parameters, pregnancy outcomes, or rates of live births.

The main objectives of treating male infertile patients with AOXs are to increase rates of pregnancy while reducing the rate of miscarriages. According to the findings, all three of the female study groups (Groups 1 and 2) had a discernible increase in the pregnancy rate among the female patients in the second ICSI trial following treatment. However, in the Second ICSI study, there was a rise in the pregnancy rate. Between the female group under examination and the previous ICSI trial. There were no statistically significant differences observed in the treatment outcomes. According to Smits *et al*. (2019), which collectively demonstrated a statistically significant increase in clinical pregnancy rates when employing different antioxidant (AOX) treatments), the results are consistent.

The study of the hormonal profile is fundamental to assessing the extent of testicular damage and if there are endocrine dysfunctions at the base. FSH, LH, and PRL are the first-level tests. FSH is the hormone that directly stimulates spermatogenesis, while LH mainly regulates the production of testosterone by the testicle. In the case of

testicular insufficiency, there is often a profile of hypogonadotropic hypogonadism. Instead, in pre-testicular forms, there are reduced levels of FSH, LH, and PRL (hypogonadotropic hypogonadism) (Kathrins and Niederberger, 2016).

Another in vivo study assessed the effect of 600 mg/day of NAC for three months to evaluate different parameters as chromatin-negative alteration induced by high oxidative stress and its consequences on sperm quality (motility, count and morphology). After this treatment, all the sperm parameters improved significantly and in parallel DNA fragmentation and protamine deficiency decreased. Positive results were also in hormonal profile, lowering FSH, LH and PRL levels and consequently increasing testosterone levels **(**Jannatifar *et al*.,2019**).** Our results show that the studied group 1 FSH, LH and PRL showed a significant increase in the second ICSI trial compared to the first ICSI.

Obesity-related hypogonadism has an adverse impact on reproductive function. Many reports suggest that, in obese men, most of the androgens are converted into estrogens, leading to a hormonal imbalance. Secondary hypogonadism specifically involves a dysfunction of the HPG axis, which regulates the production of hormones that stimulate the testes (Leisegang *et al*., 2021). Clomiphene Citrate is used as a medication for treating obesity-related hypogonadism to boost testosterone levels by increasing LH and FSH levels (Huijben *et al*., 2023). FSH, LH and PRL levels in this study displayed significantly higher levels in the second ICSI in group 2 compared with the first ICSI.

CONCLUSION

The study highlights the potential benefits of FERTITONE X antioxidant therapy on sperm motility, and improved pregnancy success. This study supports the role of antioxidants in improving male fertility. The significant improvement in pregnancy outcomes after three months of antioxidant treatment was likely attributed to a reduction in sperm DNA damage, particularly in terms of sperm DNA fragmentation.

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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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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