# Effects of Letrozole and Crocin Combination on Sperm Motility and Biochemical Markers in Azoospermia: Insights from Gene Expression Analysis

**Shahrzad Nokhbeh Zaeem**
Parand Islamic Azad University
**Mitra Heydari Nasrabadi**
heydarimitra45@piau.ac.ir
Parand Islamic Azad University
**Masoud Salehipour**
Parand Islamic Azad University
**Somayeh Ehtsham**
Parand Islamic Azad University

**Research Article**

**Keywords:** Azoospermia Crocin Busulfan Aromatase Letrozole

**Posted Date:** September 10th, 2024

**DOI:** https://doi.org/10.21203/rs.3.rs-4901570/v1



**Additional Declarations:** No competing interests reported.

# Effects of Letrozole and Crocin Combination on Sperm Motility and Biochemical Markers in Azoospermia: Insights from Gene Expression Analysis

Shahrzad Nokhbeh Zaeem[1], Mitra Heydari Nasrabadi[2], Masoud Salehipour[3]; Somayeh Ehtsham[4]

1.PhD Candidate; Faculty of Biological Sciences, Parand Islamic Azad University, Parand, Iran, shahrzad.nz1992@gmail.com

2.Associate Professor, Department of Biology, Faculty of Biological Sciences, Parand Islamic Azad University, Parand, Iran (Corresponding author) heydarimitra45@piau.ac.ir

3.Assistant Professor, Biology Department, Faculty of Biological Sciences, Parand Islamic Azad University, Parand, Iran, m.salehipour@Piau.ac.ir

4.Assistant Professor, Biology Department, Faculty of Biological Sciences, Parand Islamic Azad University, Parand, Iran, somayehtesham@gmail.com

## Abstract:

Azoospermia, characterized by the absence of sperm in ejaculation, is a severe male infertility condition resulting from defects in spermatogenesis or the testicular duct system. DNA-alkylating agents, often from chemical drugs, contribute to infertility, while antioxidants play a crucial role in addressing fertility issues in couples facing subfertility. Traditional medicinal plants, particularly saffron, have been recognized for their fertility-enhancing effects. Crocins in saffron are noted for their potential to improve fertility indicators in rat models. This study aimed to investigate the effects of the combination of letrozole and crocin on sperm motility and biochemical markers in azoospermia, complemented by gene expression analysis. Thirty male Wistar rats were divided into five groups: control, azoospermia, azoospermia with letrozole, azoospermia with crocin, and azoospermia with both letrozole and crocin. Azoospermia was induced using a 10 mg intraperitoneal injection of busulfan over ten days. Subsequent analyses included histopathological, molecular, and hormonal assessments, with data analyzed using Graph Prism software. The combination treatment with letrozole and crocin significantly improved sperm motility compared to the azoospermia group, while reducing motility compared to the control group. Additionally, there was an increase in total sperm count, Total Antioxidant Capacity (TAC), and testosterone levels, accompanied by a decrease in Total Oxidant Status (TOS) enzyme activity ($P<0.05$). QRT-PCR analysis indicated decreased expression of the GDNF gene compared to the control ($P<0.001$) but increased expression compared to the azoospermia group ($P<0.001$). Likewise, 5-alpha-reductase gene expression showed a significant decrease ($P<0.05$). Therefore, the synergistic administration of crocin and letrozole holds promise for mitigating DNA damage caused by busulfan, potentially reducing long-term damage to bone marrow and improving fertility outcomes.

**Keywords:** Azoospermia؛ Crocin؛ Busulfan؛ Aromatase؛ Letrozole.

## Introduction:

Male factors significantly contribute to infertility in couples struggling to conceive. Around 15% of people worldwide are affected by infertility [1]. Azoospermia, a severe male infertility condition, is marked by the complete absence of spermatozoa in two centrifuged semen samples [2]. Azoospermia, in contrast, involves no ejaculate being produced at all. Azoospermia affects about 1% of all men and represents 10% to 15% of male infertility cases [3]. This condition often results from various irreversible conditions affecting the testicles [4]. Azoospermia is classified into pre-testicular, testicular, or post-testicular types based on its origin. It is further divided into obstructive azoospermia (OA), where there is a blockage in the sperm-carrying ducts or vas deferens, and nonobstructive azoospermia (NOA). Differentiating these types is essential, especially for identifying primary testicular failure [5]. Sperm DNA fragmentation (SDF) is a structural alteration in DNA that leads to cellular damage and decreased viability, playing a critical role in male infertility. SDF serves as a significant prognostic and diagnostic indicator [6]. DNA damage is categorized into endogenous, caused by internal agents or chemicals, and exogenous, resulting from external factors [7]. The primary forms of endogenous DNA damage induced by reactive oxygen species (ROS) include DNA fragmentation, mitochondrial DNA damage, telomere shortening, Y-chromosome microdeletions (Y-CMs), and alterations in methylation and acetylation patterns. Thus, DNA fragmentation can affect both single-stranded and double-stranded DNA structures [8]. Chemotherapy can cross the blood-testis barrier and is known to have both short-term and long-term harmful effects on male fertility [9], with over 25% of men undergoing cancer diagnosis and treatment reporting fertility issues (ranging from mild oligozoospermia to persistent azoospermia) [10]. Busulfan is one of the most common chemotherapy drugs used before bone marrow transplantation in both children and adults [11]. Additionally, this drug is widely used in the treatment of chronic myelogenous leukemia and ovarian cancer [12]. However, busulfan also damages healthy cells during chemotherapy. The cytotoxic effects of chemotherapy drugs like busulfan induce DNA damage in cells, leading to senescence [13] and in some conditions, apoptosis [12]. This drug has the capability to induce apoptosis in germ cells [14]. On the other hand, this drug has numerous side effects on both male and female gonads, spermatogenesis, and the production of natural sperm [15]. Due to its alkylating properties, busulfan adversely affects DNA in cells with higher divisional capacity. Therefore, it exerts one of its most significant effects on testicular spermatogonia. However, not all spermatogonia are eliminated following busulfan administration [16]. Chemotherapy agents like busulfan can potentially impact hormonal balance, which may indirectly influence androgen production and metabolism. However, direct effects on 5-alpha reductase activity are less clear. As busulfan treatment has been shown to affect sperm production and function. Changes in testosterone levels due to the impact on 5-alpha reductase could also affect spermatogenesis [17]. Deficiency of 5-alpha reductase also affects male sexual

development before birth and during puberty. DHT plays an important role in male sexual development, and deficiency of this hormone disrupts the formation of external sex organs before birth [18]. Therefore, it is obvious that 5 alpha reductase is important for the development of male external genitals and male fertility. There are two isoforms of this enzyme, 5alpha reductase 1 and 2. This enzyme is highly expressed in epididymis and vesicles of semen, genital, prostate, and liver and plays the most important role in the development of the male reproductive system [19]. Reports also show that GDNF is one of the most important growth factors in spermatogonial stem cells, which promotes the proliferation of undifferentiated spermatogonies in both "in vivo" and "in vitre" [20]. GDNF is a glial derived neurotrophic factor that is produced by glial cells in the brain and other organs such as the ovary and testis (from sertoli cells) that binds to a complex receptor at the cell surface [21]. Based on an understanding of the hormonal control of spermogenesis, several drugs such as gonadotropins, androgens, estrogen receptor blockers, and aromatase inhibitors are being used as experimental treatments for idiopathic male infertility. Letrozole as an aromatase inhibitor has been widely used in the treatment of female infertility [22]. There are limited studies that have evaluated the therapeutic potential of letrozole in male infertility [23]. Yao et al. also noted that letrozole improved body weight, sperm count, motility, vitality, and plasma testosterone levels in cadmium exposed rats [24]. In another review, concluded that letrozole improved spermatogenesis but did not produce pathological changes in testicular tissue [25]. On the other hand, frequent administration of chemical drugs may directly affect the function of male gonads, leading to infertility or indirectly affect the function of the gonads, such as the prostate, semen vesicles, and Cooper gland, as well as the hypothalamus-testis axis, causing malfunction in sperm characteristics such as count, survival, motility and sperm structure [26]. This phenomenon has led researchers to turn to herbal remedies that naturally have fewer side effects [27]. Crocin has been identified as the main active biological component in saffron. In recent research, it has been revealed that saffron can increase insulin sensitivity and lower blood glucose levels and also has antioxidant properties [28]. Studies have shown that the use of natural antioxidant agents such as crocin modulates improper function caused by oxidative stress. According to studies, crocin improves mitochondrial dysfunction through signaling pathways in the endothelium, leading to reduced production of reactive oxygen species. Crocin also has a protective role against idiopathic pulmonary fibrosis [29]. Therefore, due to the increasing tendency of medical science to herbal medicines and the positive effects of saffron on the reproductive system, the aim of the present study, investigate the effects of the combination of letrozole and crocin on sperm motility and biochemical markers in azoospermia, complemented by gene expression analysis.

**Material & method:**

This study has been approved by the Ethics Committee (IR.IAU.PIAU.REC.1403.001) for the care and use of laboratory animals at the Islamic Azad University, Parand Branch in Iran.

***Animals:***

In this study, a total of 30 male Wistar desert rats (8-10 weeks old, weighing 180 to 220 grams) were obtained from the Pasteur Institute in Tehran. The rat was kept in temperature-regulated rooms with 12-hour light/dark cycles. They had unlimited access to food and water. Improvements were made in housing and monitoring the animals, and protocols were developed to ensure animal welfare. This included providing cardboard tunnels and mouse houses to enhance the cages. The mice were housed in social groups, with cage sizes meeting the legislative requirements for the number of rat [30].

***Crocin preparation:***

Crocin is a water-soluble carotenoid derived from Crocus sativus (saffron). It is formed through the esterification of the disaccharide gentiobiose with the dicarboxylic acid crocetin [31]. This compound belongs to a group of hydrophilic carotenoids, which includes monoglycosyl or diglycosyl polyene esters of crocetin [32]. In this study, crocin powder was purchased ready from Sigma Company and stored in a dry and dark place. For intraperitoneal injection, per day, crocin dissolved in distilled water with a concentration of 10 mg per milliliter and varied according to the weight of the mice [33].

***Inducing infertility in mice:***

To halt spermatogenesis and induce azoospermia in mice, a dose of 10 milligrams of busulfan was injected intraperitoneally for 10 days [34].

***Experimental design:***

The mice were randomly divided into 5 groups (each group consisting of 6 mice):

1) Control group injected with normal saline and citrate buffer intraperitoneally
2) Treatment group with a single dose of 10 mg of busulfan intraperitoneally in a volume of 1.0 milliliters for 10 days
3) Treatment group with a single dose of 10 mg of busulfan intraperitoneally in a volume of 1.0 milliliters plus a dose of 5 mg letrozole with BSF solvent
4) Treatment group with a single dose of 15 mg crocin extract with normal saline plus 10 mg of busulfan intraperitoneally in a volume of 1.0 milliliters
5) Treatment group with a single dose of 10 mg of busulfan intraperitoneally in a volume of 1.0 milliliters plus a dose of 5 mg letrozole with BSF solvent and a dose of 15 mg crocin extract with normal saline

***Sperm count and morphology:***

The epididymis was sectioned into three parts and immersed in 2.5 ml of normal saline, followed by incubation at 37 °C for 15 minutes. Subsequently, a 10 µl aliquot of the solution was placed on a Neubauer slide, and sperm within four squares were enumerated using a light microscope (Olympus BX51) equipped with a 40× objective lens. The average number of sperm counted was then recorded. The concentration of sperm per milliliter was determined utilizing the following formula: $n=\frac{(\text{mean of sperms in four square}\times 2.5}{\text{volume of one square}(100\text{nL})}$

For the assessment of abnormal sperm, a smear was prepared by placing 50 µl of the epididymal sperm solution on a slide and fixing it with 70% methanol. Papanicolaou staining was then performed. Sperm morphology was examined on each slide, and abnormal sperm (those without tails, with coiled or bent tails, or with dual and abnormal heads) were counted using an optical microscope [35]. The status of the testicular capsule tissue was also examined using trichrome Masson staining.

***Evaluation of gene expression by quantitative real-time polymerase chain reaction:***

After extraction, the testicular tissue was stored at -80°C. The tissues were homogenized thoroughly, and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany). The RNA concentration was measured using the ND1000 NanoDrop spectrophotometer. For cDNA synthesis, 2 µg of total RNA was utilized with the QuantiTect Reverse Transcription Kit (Qiagen, Germany). The quality of the synthesized cDNA was assessed via polymerase chain reaction (PCR) targeting the GAPDH gene. The PCR products were analyzed through electrophoresis on a 1% agarose gel. Primers for the reference gene, 5α-reductase, and GDNF were designed using Primer Premier 7 software (Premier BioSoft International, Palo Alto, CA, USA). The rat GAPDH gene was employed as the reference gene for data normalization (Table 1). Finally, the data were calculated using the ΔΔCt method, with GAPDH as the reference [36-37]

Table 1. List of primers for real-time PCR

| Genes | Sequence | 3'-'5 |
|---|---|---|
| r-GAPDH | F | AGGTCGGTGTGAACGGATTTG |
| | R | TGTAGACCATGTAGTTGAGGTCA |
| r-GDNF | F | CCAGCCCAGAGAATTCCAGAG |
| | R | GCTTCACAGGAACCGCTACAA |
| r-5α-reductase | F | TCATATGTGTGTGTTGGGGGT |
| | R | ACATTTCTCCTGATGCTCCCC |

***Biochemical analyses:***

***Enzyme Activity of Oxidative and Antioxidative Status:***

The oxidative and antioxidative status of liver tissue was measured using a commercial TOS/TAC kit from Biotech, employing spectrophotometric and colorimetric methods. For this purpose, 100 milligrams of tissue were placed in one milliliter of RIPA buffer and centrifuged at 4000 RPM for 20 minutes. After preparing the solutions, the samples were aliquoted into a 96-well microplate [38]. To measure total antioxidant enzyme activity, 50 microliters of the sample were added to each well, followed by the addition of 200 microliters of working buffer solution. After incubating for 10 minutes, the absorbance was read at 560 nanometers. For assessing the stability of oxidative status, 10 microliters of the sample were added to the well. Initially, 190 microliters of chromogenic solution were added, and the absorbance was read at 490 nanometers. Subsequently, after adding 50 microliters of Solution 3, the mixture was incubated for 2 minutes. Finally, 50 microliters of stop solution were added, and the absorbance was measured again at 490 nanometers [39].

***Measurement of testosterone hormone:***

The collected blood samples were centrifuged to separate the serum. Testosterone levels were quantitated using the Free Testosterone kit from Pars Peyvand Company. In this assay, blood samples were initially added to the wells, followed by the introduction of testosterone solution and anti-testosterone solution. The samples were then incubated at 37°C for 90 minutes. After incubation, the wells were washed with deionized water, and a substrate was added to each well, followed by an additional incubation period of 20 minutes. The reaction was subsequently terminated by adding a stop solution, and the absorbance was measured at a wavelength of 450 nanometers [40].

***Statistical analysis:***

The data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare the means in the study groups ($P < 0.05$), and statistically significant differences were considered. The graphs were also plotted using Graph Pad Prism software.

**Result:**

***Sperm motility***

Sperm motility in the azoospermia group treated with letrozole and crocin exhibited a significant decrease when compared to the control group ($P<0.001$). However, this group also demonstrated a substantial increase in motility relative to the untreated azoospermia group ($P<0.0001$). These results indicate that while the combination treatment reduced

motility compared to healthy controls, it enhanced motility compared to azoospermia alone. Thus, the effects of letrozole and crocin are nuanced, leading to improvements over the untreated condition. Overall, these findings highlight the complex interaction between treatment and sperm motility dynamics-( Figure-1).

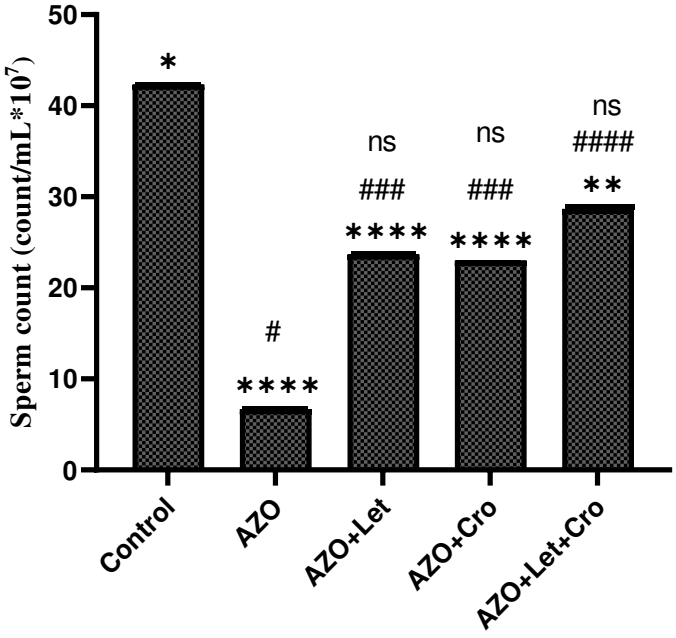



Figure 1-Sperm motility rate in the studied groups.

*: Significant differences with the control group (P<0.0001, P<0.005)

#: Significant difference with azoospermia group (P<0.001 , P<0.0001)

ns: Significant difference with azoospermia + letrozole group (P>0.05)

***Sperm viability***

Sperm viability in the azoospermia groups treated with crocin or letrozole did not demonstrate significant changes. In contrast, the azoospermia group showed a marked increase in sperm viability relative to the control group. This difference was statistically significant (P<0.05). Thus, while the treatments did not impact sperm viability, the azoospermia group displayed improved viability when compared to controls. These findings suggest a specific response within the azoospermia context.– ( Figure-2).

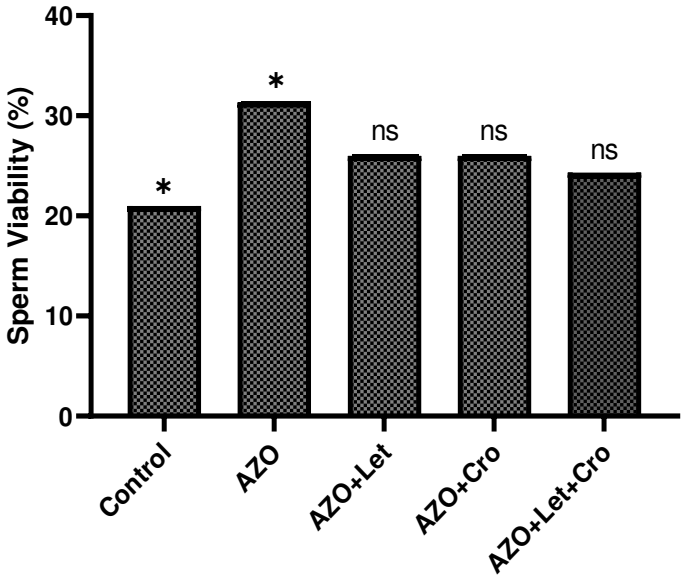



Figure 2- Sperm viability rate in the studied groups.

* Significant difference at significance level >0.05, ns no significant difference

### *Total sperm count*

The total sperm count in the azoospermia group receiving treatment with letrozole and crocin showed a significant decrease compared to the control group (P<0.001). Conversely, this treated group exhibited a notable increase in sperm count relative to the untreated azoospermia group (P<0.0001). These findings suggest that while the combination treatment resulted in lower sperm numbers than the control, it did improve count compared to the untreated condition. Thus, letrozole and crocin may contribute to enhancing sperm production in an azoospermia context. Overall, the data highlight the differential impact of treatment on sperm count dynamics– ( Figure-3).

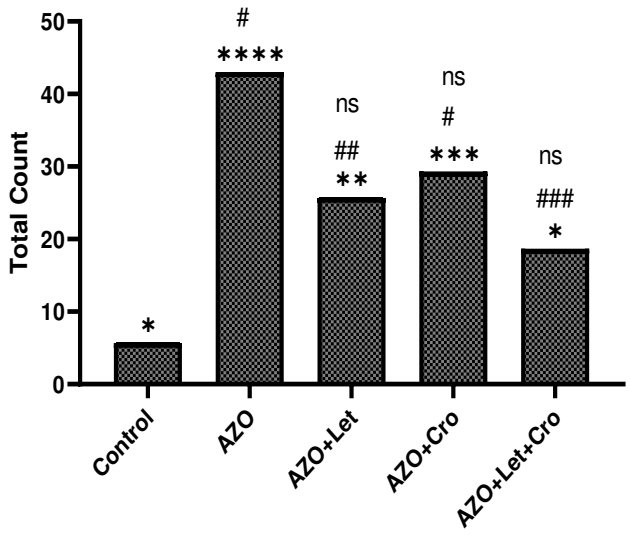



Figure 3- Total sperm count in the studied groups.

*: Significant differences with the control group (P<0.0001, P<0.005)

#: Significant difference with azoospermia group (P<0.001, P<0.0001)

ns: Significant difference with azoospermia + letrozole group (P>0.05)

### *Testicular capsule*

The examination of the testicular capsule in the azoospermia group treated with letrozole revealed significant evidence of fibrotic changes. In this group, fibroblastic cells prominently produced type I collagen fibers, leading to a notable increase in the diameter of the capsule compared to the other two groups. The thickness of the testicular capsule, as revealed by specific trichrome staining, was clearly distinguishable from surrounding tissue structures. Additionally, in the azoospermia group treated with both letrozole and crocin, there were observable changes, including the dilation and tortuosity of adjacent vessels beneath the testicular capsule. These vascular alterations were markedly different from those seen in the azoospermia + crocin and azoospermia groups, suggesting a significant manifestation of fibrotic processes in the letrozole-treated group-(Figure-4).

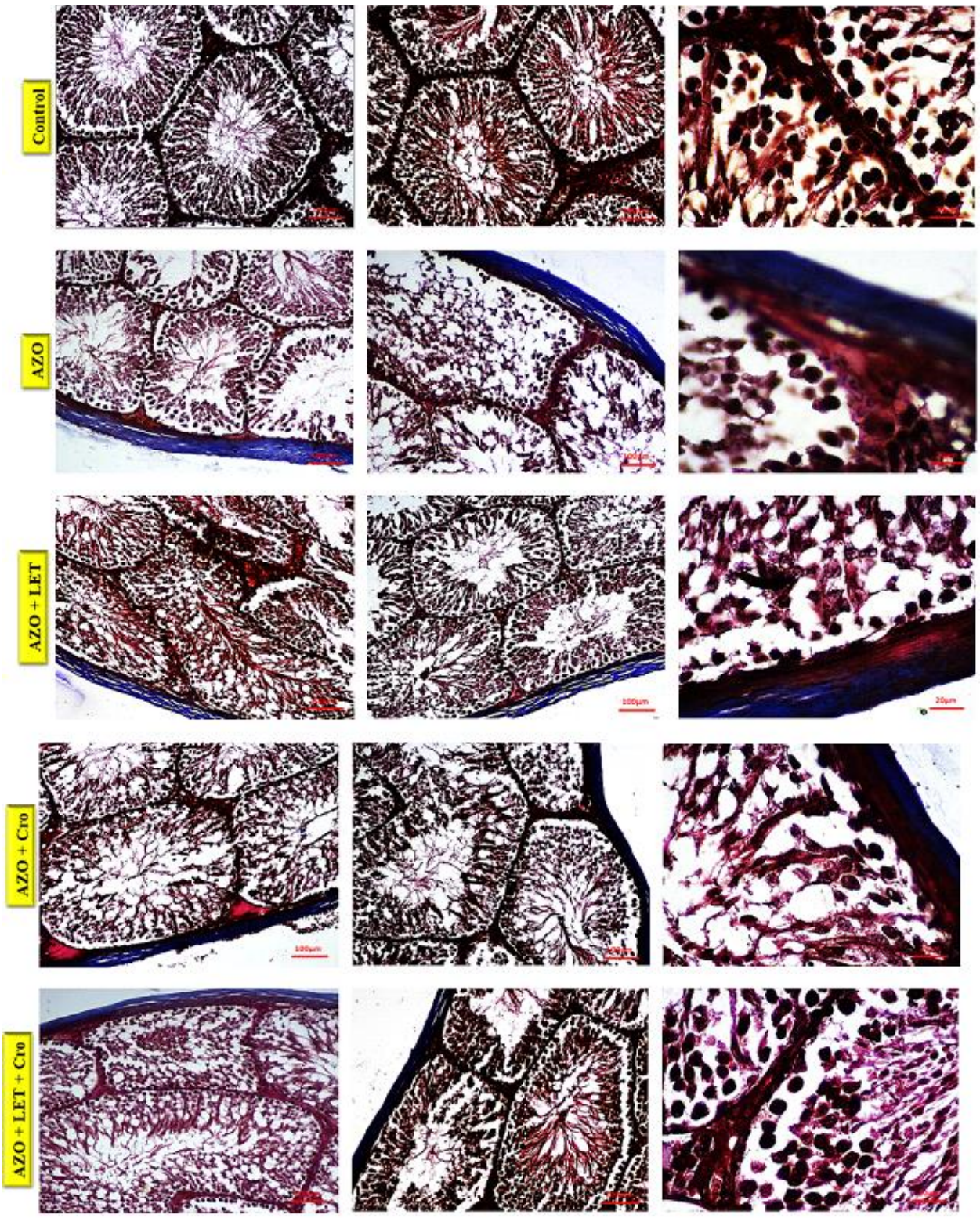



Figure-4: The results of Masson's trichrome staining of the testicular capsule.

Control: Image of testicular tissue from control group rats showing normal morphology; Azo: Image of testicular tissue from rats receiving busulfan drug at a dose of 10 mg/kg; Azo+Let: Image of testicular tissue from rats

receiving busulfan drug at a dose of 10 mg/kg along with Letrozole drug; Azo+Cro: Image of testicular tissue from rats receiving busulfan drug at a dose of 10 mg/kg along with crocin; Azo+Let1+Cro: Image of testicular tissue from rats receiving busulfan drug at a dose of 10 mg/kg along with Letrozole and crocin simultaneously.

### *Genes expression:*

Based on the results obtained from the $2^{-\Delta\Delta ct}$ method of qRT-PCR and two-way ANOVA analysis, the expression level of the 5-alpha-reductase gene in the azoospermia group was significantly reduced compared to the control group and other groups ($P<0.001$). Additionally, the Azoospermia + Letrozole and Azoospermia + Crocin groups showed a significant decrease compared to the control group, with the reduction being more pronounced in the Azoospermia + Letrozole group ($P<0.001$). Furthermore, the Azoospermia + Letrozole + Crocin group exhibited a significant increase in the expression of the 5-alpha-reductase gene compared to the azoospermia group ($P<0.001$). The Azoospermia + Letrozole group did not show a significant difference compared to the azoospermia group ($P>0.05$). In intergroup comparisons, the Azoospermia + Letrozole + Crocin group demonstrated a significant increase in the 5-alpha-reductase gene expression compared to the Azoospermia + Letrozole and Azoospermia + Crocin groups ($P<0.005$) (Finger-5). The expression level of the GDNF gene in the azoospermia group was significantly reduced compared to the control group and other groups ($P<0.001$). Additionally, the Azoospermia + Letrozole and Azoospermia + Crocin groups showed a significant decrease compared to the control group, with the reduction being more pronounced in the Azoospermia + Letrozole group ($P<0.001$). Furthermore, the Azoospermia + Letrozole + Crocin group exhibited a significant increase in the expression of the GDNF gene compared to the azoospermia group ($P<0.001$). The Azoospermia + Letrozole group did not show a significant difference compared to the azoospermia group ($P>0.05$). In intergroup comparisons, no significant differences in GDNF gene expression were observed between the Azoospermia + Letrozole, Azoospermia + Crocin, and Azoospermia + Letrozole + Crocin groups ($P>0.05$) (Finger-5).

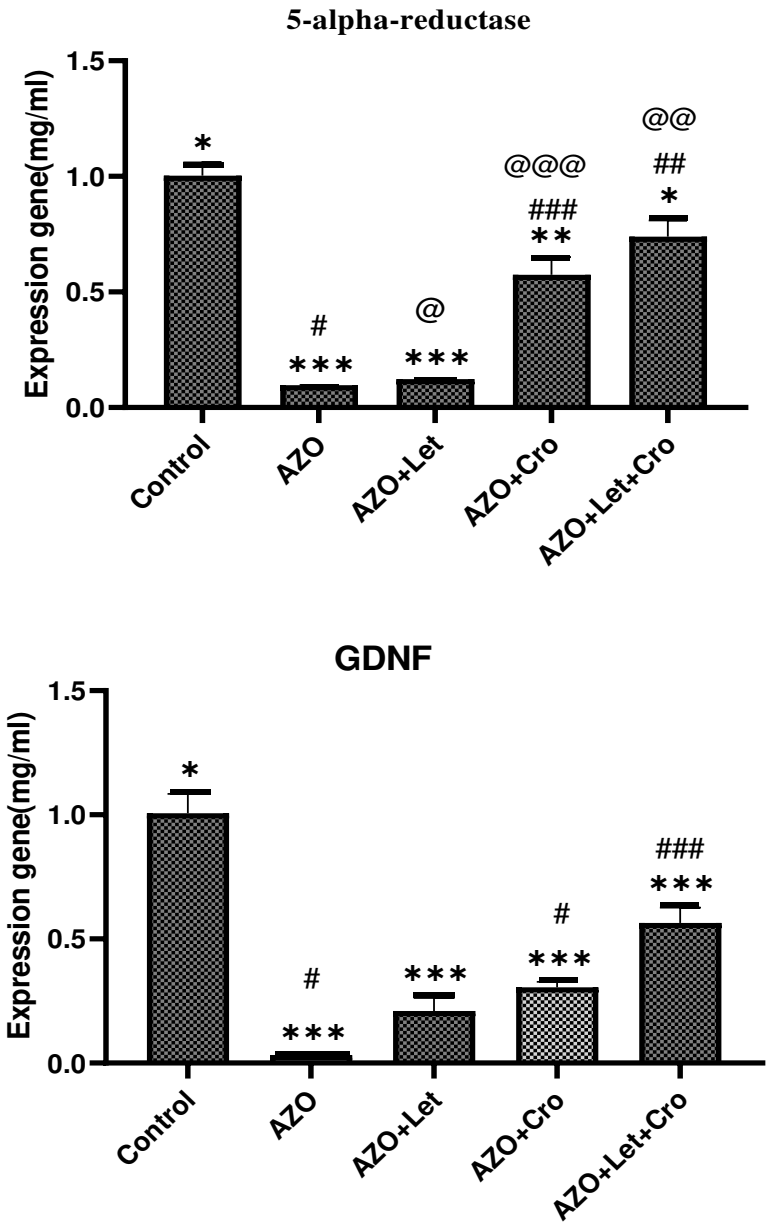



Figure -5 The results obtained from the expression levels of the genes GDNF & 5-alpha-reductase in different groups.

*: Significant differences with the control group (P<0.0001, P<0.005, P<0.5)

#: Significant difference with azoospermia group (P<0.001, P<0.0001)

@: Significant difference with azoospermia + letrozole group (P<0.001,P<0.01,P<0.005)

***Activity of Oxidative and Antioxidative Enzymes:*** Based on the results obtained from the current study, the total antioxidant enzyme activity (TOS) in the group (Azoospermia + Letrozole + Crocin) showed a significant decrease

compared to the azoospermia group (P<0.05); however, no significant changes were observed in the total oxidant enzyme (TAC). The TOS enzyme activity in the Azoospermia + Crocin group increased compared to the control group (P<0.005) and decreased significantly when compared to the azoospermia group (P<0.001). Conversely, TAC enzyme activity in the Azoospermia + Crocin group decreased compared to the control group and showed a significant increase compared to the azoospermia group (P<0.005). In the Azoospermia + Letrozole group, the TOS enzyme activity increased compared to the control group (P<0.05) but exhibited a significant decrease compared to the azoospermia group (P<0.001). However, TAC enzyme activity was significantly reduced compared to the control group (P<0.05) (Figure-6).

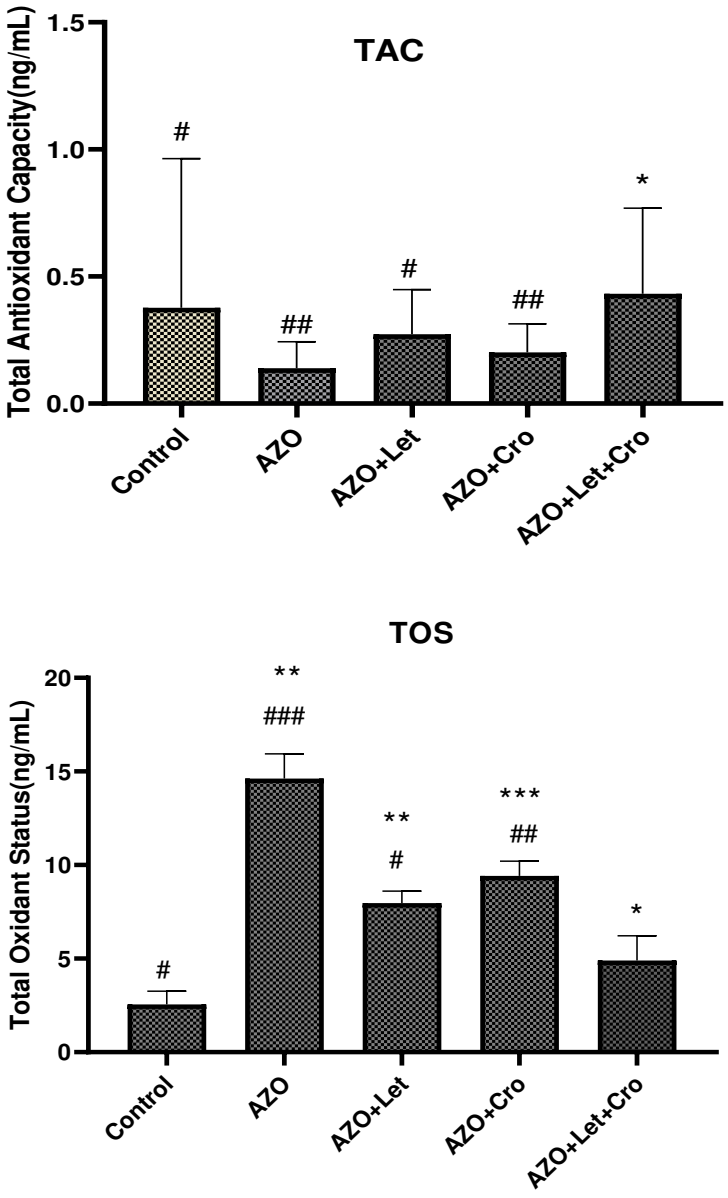


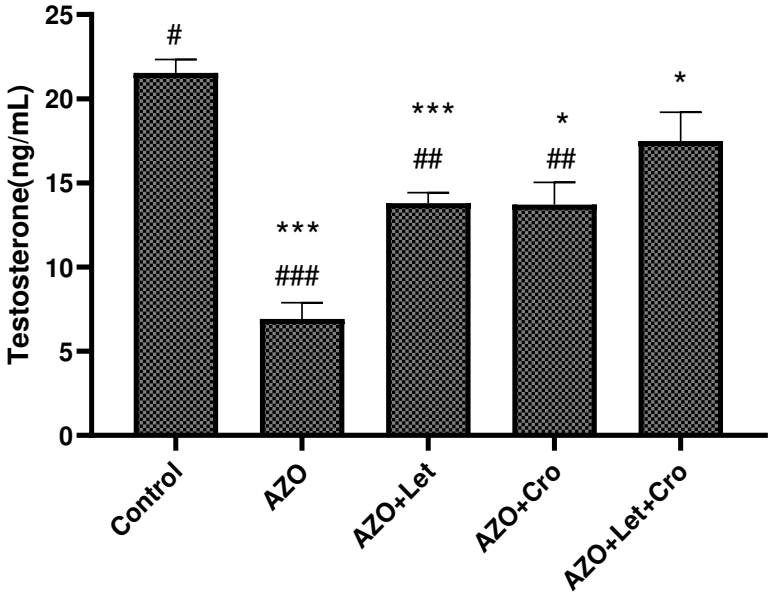
Figure -6 The results obtained from the measurements of total oxidant enzyme activity (TAC)& total antioxidant enzyme activity (TOC) compared to the control group, the azoospermia group, and the Azoospermia + Letrozole group.

*: Significant differences with the control group (P<0.0001, P<0.005, P<0.5)

#: Significant difference with azoospermia group (P<0.001, P<0.0001)

### *Testosterone hormone:*

The level of testosterone hormone in the azoospermia group was significantly reduced compared to the control group (P<0.001). Meanwhile, the testosterone levels in the Azoospermia + Letrozole and Azoospermia + Crocin groups showed a significant decrease compared to the control group (P<0.005). Additionally, when comparing the intergroup differences with the azoospermia group, the testosterone level in the Azoospermia + Letrozole + Crocin group exhibited a significant increase (P<0.001). In contrast, both the Azoospermia + Letrozole and Azoospermia + Crocin groups showed a similar increase (P<0.05) compared to the azoospermia group (Figure-7).




Finger-7 -The results of the variations in testosterone hormone levels in different groups.

*: Significant differences with the control group (P<0.0001, P<0.005, P<0.5)

#: Significant difference with azoospermia group (P<0.001, P<0.0001)

## Discussion:

This study was conducted with the aim of reducing or eliminating the complications arising from the drug busulfan on the mechanisms of azoospermia. The effects were evaluated through various assessments involving the simultaneous administration of rats with crocin and letrozole, which will be elaborated upon in the following sections. Sperm morphology

and semen parameters are considered primary morphological and physicochemical diagnostic markers of male infertility. These factors are crucial for the development of appropriate treatment strategies [41]. In examining sperm parameters, combined treatment with letrozole + crocin caused a significant decrease in sperm motility compared to the control group and a significant increase compared to the azoospermia group, but in sperm viability retention did not show any significant differences with other groups. While the total sperm count in the group treated with letrozole + crocin decreased compared to the azoospermia group treated with letrozole and significant increased compared to the control group. These results are consistent with the previous reports [42–43]. This suggests that while this treatment may have potential effects, it could also impair the motility of sperm cells under certain conditions [44]. Conversely, the treatment demonstrated a significant improvement in sperm motility compared to the azoospermia group, indicating that it may help rejuvenate or enhance motility in cases of azoospermia, at least in terms of the comparative assessment [45]. This finding is particularly important as it suggests that while the treatment may reduce motility in a general sense, it can still offer benefits in specific pathological scenarios [46]. However, when it comes to sperm viability, the study found that there were no significant differences across the various treatment groups. This lack of difference implies that while motility may be affected by the combined treatment, the overall survival of sperm cells does not vary significantly when treated with letrozole and crocin compared to other groups. Maintaining sperm viability is crucial for fertility, and the results of this study highlight the need for further research to clarify the complexities of how such treatments can influence different sperm parameters [47]. Future studies should also aim to explore the underlying mechanisms contributing to these observed effects on motility and viability, which could help in optimizing treatment protocols for male infertility. Gregoriou et al. [48] reported in a study that all sperm parameters increase after treatment with letrozole due to the rise in gonadotropin and testosterone levels. In many cases of spermatogenic situations, letrozole treatment improves the number of motile sperm by increasing the levels of gonadotropin and serum testosterone [49].Crocin is believed to enhance sperm motility by increasing intracellular calcium levels. Calcium ions play a crucial role in various cellular processes, including muscle contraction and motility. In sperm, an increase in intracellular calcium can lead to improved flagellar movement, which is essential for sperm motility [50]. Also, crocin may also influence the cyclic adenosine monophosphate (cAMP) signaling pathway. cAMP is a secondary messenger that regulates various physiological processes, including sperm motility. By increasing cAMP levels, crocin can inhibit the activity of phosphodiesterase, an enzyme that breaks down cAMP. This inhibition can lead to prolonged cAMP signaling, enhancing sperm motility [51-52]. Given the limited number of cytoplasmic enzymes in sperm cells, the ability to repair oxidative damage is severely constrained [53]. In this regard, antioxidants and antioxidant enzymes present in seminal fluid are of special importance and play a key role in defending against oxidation-induced damage. The results of a study conducted by

Mesbahzadeh *et al.* [54] confirm these findings and indicate that the presence of crocin can assist in the motility of immotile sperm. Furthermore, according to a report by Mehdipour *et al.* [55], the addition of crocin to the culture medium improves the viability of rooster sperm. Additionally, other research has shown that the use of crocin can lead to a reduction in the levels of free radicals and, ultimately, enhance the viability of sperm [56]. Rueangchainikhom et al. [57] concluded that crocin supplementation is effective on sperm permetries, while El-Sheikh *et al.* [58] reported the protective effect of crocin on the AKT/FOXO pathway against testicular toxicity caused by ionizing radiation. Also, in other studies, it has been found that the use of crocin reduces free radical levels and ultimately increases sperm viability [59]. In this study, the increase in testosterone levels observed in the treated group is significant as testosterone is critical for many aspects of male health, including libido, energy levels, and the maintenance of muscle mass. Moreover, testosterone plays a pivotal role in sperm production. Therefore, higher testosterone levels could potentially lead to improved sperm count and motility, offering a promising avenue for treating male infertility, particularly in cases classified as azoospermia [60-61]. crocin vessel dilation and increased blood flow, in addition to its antioxidant properties, appear to be a factor in the increase in testosterone levels in the current study [62]. In fact, testosterone controls the secretion of the LH hormone from the anterior pituitary through the negative feedback mechanism. Increased serum testosterone levels from treatment with crocin indicate the effect of crocin on the hypothalamus-pituitary axis of the testicle [63]. It seems that crocin as one of the main compounds of saffron can enhance the sexual performance of animal models and increase testosterone levels and play a prominent role in improving Sperm quality plays. In general, crocin has protective effects that improve some of the effects of bosulfan-induced azoospermia in reducing sperm count and sperm abnormalities [64]. Because, busulfan in single doses higher than 10 mg/kg exerts numerous side effects on the gonads of both sexes, spermatogenesis and natural sperm production [65]. According to the investigations carried out, busulfan has harmful effects on the fertility of mice and leads to azoospermia [66].

Recent studies on stromal cells have shown that the myoid surrounding a tube is an important source of GDNF in the retinal testicle [67], while rats have reported an over-expression of GDNF ‹Clusters show undifferentiated spermatogonia [68]. The results of the molecular analysis indicate that the expression level of the GDNF (Glial-Derived Neurotrophic Factor) gene exhibited a significant decrease when compared to a control group but showed a significant increase relative to the azoospermia group. A significant decrease in GDNF expression in the tested group compared to a control suggests an impairment in the mechanisms supporting spermatogenesis or testicular function. This finding may indicate that the physiological or treatment conditions applied to the study group negatively impacted the regulation of the GDNF gene. It can point toward underlying issues such as oxidative stress, hormonal imbalances, or disruptions in the

microenvironment of the testes. Conversely, an increase in GDNF expression when compared to the azoospermia group indicates a potential compensatory mechanism or recovery due to the treatment or environmental changes encouraging enhanced spermatogenesis. This result suggests that the interventions tested could be beneficial in terms of enabling the expression of GDNF, which is essential for supporting sperm development and potentially improving fertility outcomes. In Khan et al.'s research, it was shown that crocin improves gene expression related to fertility and fertility potential of buffalo sperm [69]. Spermatogonia of type A have the potential to modulate their homeostasis by downregulating GDNF expression when they are sufficiently abundant. This finding aligns with observations in normal mice, where the removal of germ cells after exposure to busulfan results in an upregulation of GDNF levels [70]. Garcia *et al.* also reported in their research that the mRNA expression of the GDNF gene and the levels of GDNF protein per Sertoli cell were significantly decreased in SCO testes [71].

The molecular analysis conducted in the presented study revealed a significant decrease in the expression levels of the 5α-reductase gene when compared to the control group. A reduction in 5α-reductase expression could lead to alterations in the androgen environment, potentially impacting sperm development and maturation and affect the composition of seminal plasma, possibly influencing fertility [72-73]. on the other hand, impact of Letrozole on increased testosterone levels might lead to higher 5α-reductase expression, thereby increasing DHT levels. Elevated DHT can enhance spermatogenesis, which could be beneficial for men experiencing azoospermia due to low DHT levels [74]. Any improvement in androgen signaling due to letrozole treatment could potentially reverse azoospermia in certain contexts [75]. By reducing oxidative damage in Leydig and Sertoli cells (important cells in the testes), crocin could help maintain or enhance testosterone production, potentially influencing 5α-reductase activity and expression [76]. While some studies suggest that certain compounds derived from natural sources can inhibit 5α-reductase, there is limited direct evidence regarding crocin's specific inhibitory effects. However, if crocin does exhibit such actions, it could help to modulate DHT levels in specific contexts [77]. Also, administration of crocin may lead to improvements in various semen parameters, including sperm count and motility. If crocin helps increase the proliferation and function of Sertoli cells and enhances testosterone-mediated spermatogenesis, this could indirectly suggest a supportive role for 5α-reductase [78].

The activity of the TAC enzyme and testosterone hormone in the group treated with Letrozole and Crocin increased significantly compared to the azoospermia group. In groups treated with Letrozole or Crocin alone, the activity levels decreased significantly compared to the control group. The combination treatment demonstrated a synergistic effect, significantly increasing the activity of TAC and levels of testosterone compared to the azoospermia group. This suggests that Letrozole and Crocin together enhance antioxidant capabilities and possibly improve endocrine function, contributing to potentially better

spermatogenic outcomes. Also, the activity of the TOS enzyme decreased significantly in the group treated with Letrozole and Crocin compared to the azoospermia group. The significant reduction in TOS enzyme activity in the group treated with Letrozole and Crocin indicates reduced oxidative stress. Decreasing oxidative stress can be beneficial as it reduces cellular damage and improves the overall testicular environment, which is crucial for spermatogenesis. In this regard, Salhshoor *et al.* reported that crocin possesses antioxidant and defensive effects, enhancing the quality of certain sperm by improving morphology, viability, motility, and normal count [56]. Additionally, the positive effects of crocin on many sperm parameters have been attributed to the increase in testosterone levels as a result of crocin injection [79]. Moreover, crocin can mitigate the negative effects of oxidative stress and augment cellular capacity to overcome oxidative stress conditions by preventing glutathione depletion and enhancing antioxidant capacity [80]. The findings of Safayedgar *et al.* were consistent with the results of the present study, indicating that phytoestrogens like crocin bind to testicular estrogen receptors and stimulate spermatogenesis through strategies such as increasing epithelial layers and the diameters of seminiferous tubules and lumens [81]. In other studies, the findings of Asadi *et al.* demonstrated that saffron significantly improves the epididymal sperm parameters exposed to cadmium [82].

The interaction between letrozole and antioxidant enzyme activity has potential implications for both cancer treatment and fertility [83]. Optimizing letrozole use in women with oxidative stress-related infertility could enhance reproductive outcomes by balancing hormone levels and antioxidant activity [84]. Clinical studies have shown the efficacy of letrozole in enhancing ovulation rates in women with polycystic ovary syndrome (PCOS) and other forms of ovulatory dysfunction [85]. Compared to clomiphene citrate, letrozole increases the pregnancy rate for ovulation in women with unexplained infertility [86]. Therefore, letrozole is a potent and selective aromatase inhibitor that inhibits intracellular aromatase activity. As a result, it can reduce the rate of conversion of testosterone to estrogen and estrone to androstenedione, subsequently decreasing the negative feedback to the hypothalamus through the inhibition of aromatization [87]. This leads to an increase in FSH levels due to the activation of the pituitary axis, as well as an increase in luteinizing hormone levels, which in turn can enhance spermatogenesis and testosterone levels [88].

Oxidative stress can result in breaks in DNA strands, which negatively affects the fertilization ability of sperm [89]. This type of fragmentation is associated with abnormalities in embryos, which can disrupt their development and raise the chances of early pregnancy loss [90]. Beyond fragmentation, oxidative stress can induce genetic mutations and chromosomal abnormalities within sperm DNA. These irregularities can contribute to developmental defects in embryos and increase the risk of miscarriage [91]. The DNA found in sperm cells is particularly susceptible to oxidative damage due to its high level of compaction and tight packaging, making it vulnerable to reactive oxygen

species (ROS) [92]. This type of damage has been associated with lower fertilization rates, diminished embryo quality, and a greater likelihood of developmental issues in offspring [93]. The positive effects of crocin on sperm parameters suggest its potential as a complementary therapy for male infertility [94]. It might be particularly beneficial in individuals with oxidative stress-related fertility issues [95]. The mechanisms through which crocin exerts its effects include modulation of enzymatic activities and gene expression related to antioxidant defense and reproductive health. Preliminary studies suggest that crocin can enhance spermatogenesis by promoting the proliferation of germ cells within the testes [96]. This is particularly crucial for patients with azoospermia, where sperm production is disrupted [97]. Chronic inflammation can contribute to azoospermia by impacting testosterone production and spermatogenesis [98]. Crocin's anti-inflammatory effects may mitigate these inflammatory processes, helping restore normal function [99]. Considering that preliminary findings are promising, further clinical trials are necessary to fully understand the efficacy and optimal dosing of crocin for enhancing sperm parameters in various populations. Thus, azoospermia caused by busulfan can lead to long-term fertility issues. In many cases, men who undergo treatment may not recover normal spermatogenesis after cessation of therapy. Even those who regain sperm production may develop oligospermia (reduced sperm count) rather than complete recovery to normal sperm production levels. The combination of letrozole, an aromatase inhibitor commonly used for the treatment of hormone receptor-positive breast cancer and for inducing ovulation, alongside crocin a natural compound derived from saffron with antioxidant properties could yield beneficial effects on fertility. Therefore, carotenoids in saffron can directly or indirectly regulate epigenetic changes and change gene expression profiles. Among the limitations of the research, one can refer to the lack of sample studies and the mortality of mice during the testing process, which seems essential to address through further studies and a focus on the precise dosage of crocin and attention to the related mechanisms of action.

## Conclusion

The simultaneous treatment of letrozole and crocin demonstrates a notable potential in mitigating the effects of busulfan-induced DNA damage. Busulfan, an alkylating agent known for its DNA-damaging properties, poses significant risks not only to rapidly dividing cancer cells but also to normal somatic and germ cells, leading to detrimental reproductive outcomes. In this context, letrozole, an aromatase inhibitor, appears to synergistically interact with crocin, a bioactive compound derived from saffron, which possesses antioxidant properties. This combination treatment may work by both enhancing protective cellular mechanisms and reducing oxidative stress associated with busulfan exposure. The results indicate that the dual administration of letrozole and crocin could effectively reduce the extent of DNA damage in cells influenced by busulfan, as observed through biochemical markers and molecular analyses. This treatment could potentially

restore normal cellular function and fertility parameters that are commonly disrupted by chemotherapy agents. Further investigation is essential to fully characterize the mechanisms through which this combined therapy supports DNA integrity and to evaluate its potential for protecting against reproductive toxicity in clinical settings. Such insights may lead to improved strategies for preserving fertility in patients undergoing chemotherapy, ultimately enhancing their quality of life post-treatment.

**Data availability:**

The data underlying this article will be shared upon reasonable request to the corresponding author.

**Abbreviations**

| | |
|---|---|
| Azo | Azoospermia |
| Let | Letrozole |
| OA | Obstructive mechanisms |
| NOA | Non-obstructive mechanism |
| ROS | Reactive oxygen species |
| AIs | Aromatase inhibitors |
| DHT | Di hydro testosterone |
| PCR | Polymerase chain reaction |
| TAS | Total antioxidant capacity |
| TOC | Total oxidant capacity |
| SDF | Sperm DNA fragmentation |
| Y-CMs | Y-chromosome microdeletions |
| cAMP | cyclic adenosine monophosphate |
| PCOS | polycystic ovary syndrome |

**References:**


1. Sharma, A., Minhas, S., Dhillo, W. S., & Jayasena, C. N. (2021). Male infertility due to testicular disorders. Journal of Clinical Endocrinology and Metabolism, 106 (2), e442–e459. [PMC free article] [PubMed] [CrossRef] [Google Scholar]
2. Jarvi K, Lo K, Grober E, Mak V, Fischer A, Grantmyre J, Zini A, Chan P, Patry G, Chow V, Domes T. The workup and management of azoospermic males. Can Urol Assoc J. 2015 Jul-Aug;9 (7-8):229-35. [PMC free article] [PubMed]
3. Köse, S., Yersal, N., Önen, S., Korkusuz, P. (2018). Comparison of hematopoietic and spermatogonial stem cell niches from the regenerative medicine aspect. Adv Exp Med Biol, 1107, 15–40. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

4. Köse, S., Aerts-Kaya, F., Köprü, Ç. Z., Nemutlu, E., Kuşkonmaz, B., Karaosmanoğlu, B., Taşkıran, E. Z., Altun, B., Uçkan Çetinkaya, D., & Korkusuz, P. (2018). Human bone marrow mesenchymal stem cells secrete endocannabinoids that stimulate in vitro hematopoietic stem cell migration effectively comparable to beta-adrenergic stimulation. Experimental Hematology, 57, 30-41.e31. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

5. Salem, M., Khadivi, F., Javanbakht, P., Mojaverrostami, S., Abbasi, M., Feizollahi, N., Abbasi, Y., Heidarian, E., & Rezaei Yazdi, F. (2023). Advances of three-dimensional (3D) culture systems for in vitro spermatogenesis. Stem Cell Research & Therapy, 14 (1), 262. [PMC free article] [Google Scholar]

6. Le, M.T.; Nguyen, T.A.T.; Nguyen, H.T.T.; Nguyen, T.T.T.; Nguyen, V.T.; Le, D.D.; Nguyen, V.Q.H.; Cao, N.T. Does sperm DNA fragmentation correlate with semen parameters? Reprod. Med. Biol. 2019, 18, 390–396. [PubMed] [CrossRef] [Google Scholar]

7. Björndahl L, Kirkman Brown J. The sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen: ensuring quality and standardization in basic examination of human ejaculates. Fertil Steril. 2022;117 (2):246–51. [CrossRef] [Google Scholar]

8. Pallotti, F.; Pelloni, M.; Faja, F.; Di Chiano, S.; Di Rocco, A.; Lenzi, A.; Lombardo, F.; Paoli, D. Semen Quality in Non-Hodgkin Lymphoma Survivors: A Monocentric Retrospective Study. Hum. Reprod. 2021, 36, 16–25. [Google Scholar] [CrossRef]

9. Vakalopoulos, I.; Dimou, P.; Anagnostou, I.; Zeginiadou, T. Impact of Cancer and Cancer Treatment on Male Fertility. Hormones 2015, 14, 579–589. [Google Scholar] [CrossRef] [PubMed]

10. Delessard, M.; Saulnier, J.; Rives, A.; Dumont, L.; Rondanino, C.; Rives, N. Exposure to Chemotherapy During Childhood or Adulthood and Consequences on Spermatogenesis and Male Fertility. Int. J. Mol. Sci. 2020, 21, 1454. [Google Scholar] [CrossRef] [PubMed]

11. Anjamrooz SH, Movahedin M, Mowla SJ, Bairanvand SP. Assessment of morphological and functional changes in the mouse testis and epididymal sperms following busulfan treatment. Iran Biomed J. 2007; 11: 15-22. [PubMed] [CrossRef] [Google Scholar]

12. Temitope AA, Tolulope OA, Funlola CT, Khaleed TB, et al. Protective Effect of Tahitian Noni Juice on the Reproductive Functions of Male Wistar Rats Traeted with Cyclophosphamide. Iran J Pharmacol Ther. 2011; 10(1): 39-43. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

13. Nieto Y, Thall P, Valdez B, Andersson B, et al. High-Dose Infusional Gemcitabine Combined with Busulfan and Melphalan with Autologous Stem-Cell Transplant in Patients with Refractory Lymphoid Malignancies. Biol Blood Marrow Transplant. 2012; 18(11): 1677–1686. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

14. Mohammad Ghasemi F, Bahadori MH, Faghani M, Nasiri E, et al. Buserelin Inhibits Apoptosis in Male Germ Cells Induced by Busulfan in Mouse Testis. J of Iran Anat Sci. 2009; 7: 45.[PubMed] [CrossRef] [Google Scholar]

15. Mohamad-Ghasemi F, Faghani M, Fallahkarkan M. The protective effect of melatonin on sperm parameters, epididymis and seminal vesicle morphology in adult mouse treated with busulfan. J Iran Anat Sci. 2010; 8: 25-36. [CrossRef] [Google Scholar]

16. Mohammadghasemi F, Soleimanirad J, Ghanbari AA. An Ultrastructural Study on the Apoptotic Features of Spermatogenic Cells following Busulfan Treatment in Adult Mice. J Reprod Infertil. 2008; 8(4): 319-329. [CrossRef] [Google Scholar]

17. Iwamoto T., Hiraku Y., Oikawa S., Mizutani H., Kojima M., Kawanishi S. DNA Intrastrand Cross-Link at the 5'-GA-3' Sequence Formed by Busulfan and Its Role in the Cytotoxic Effect. *Cancer Sci.* 2004;95:454–458. [PMC free article] [PubMed] [CrossRef] [Google Scholar] [Ref list]

18. Bibancos M, Cavagnoli M, Bonetti TC, Semaco E, Motta EL, Serafini PC. Letrozole Therapy for Obstructive Azoospermic Men before in vitro Fertilization (IVF) treatment with Percutaneous Epididymal Sperm Aspiration. JBRA Assist Reprod. 2015 Nov 1;19(4):230-4. [CrossRef] [Google Scholar]

19. Kooshesh L, Bahmanpour S, Zeighami S, Nasr-Esfahani MH. Effect of Letrozole on sperm parameters, chromatin status and ROS level in idiopathic Oligo/Astheno/Teratozoospermia. Reprod Biol Endocrinol. 2020 May 13;18(1):47. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

20. Wright WW. The Regulation of Spermatogonial Stem Cells in an Adult Testis by Glial Cell Line-Derived Neurotrophic Factor. Front Endocrinol (Lausanne). 2022 Jun 3;13:896390. [CrossRef] [Google Scholar]

21. Mao Y, Li D, Yang Q, Pei X, Duan Z, Ma M. Prenatal BPA exposure disrupts male reproductive functions by interfering with DNA methylation and GDNF expression in the testes of male offspring rats. Environ Sci Pollut Res Int. 2023 Apr;30(18):53741-53753. [PMC free article][CrossRef] [Google Scholar]

22. Diamond MP, Legro RS, Coutifaris C, Alvero R, Robinson RD, Casson P, Christman GM, Ager J, Huang H, Hansen KR, Baker V, Usadi R, Seungdamrong A, Bates GW, Rosen RM, Haisenleder D, Krawetz SA, Barnhart K, Trussell JC, Ohl D, Jin Y, Santoro N, Eisenberg E, Zhang H; NICHD Reproductive Medicine Network. Letrozole, Gonadotropin, or Clomiphene for Unexplained Infertility. N Engl J Med. 2015 Sep 24;373(13):1230-40. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

23. Yao Y, Wan Y, Shi X, Guo L, Jiang H, Zhang X, Xu B, Hua J. Letrozole protects against cadmium-induced inhibition of spermatogenesis via LHCGR and Hsd3b6 to activate testosterone synthesis in mice. Reprod Biol Endocrinol. 2022 Mar 2;20(1):43. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

24. Hmood Jassim A, Abed AS, Kareem Thamer A, Judi HK, Enad AH. Effect of Letrozole Administration as a Selective Aromatase Inhibitor on Male Rat's Reproductive Performance. Arch Razi Inst. 2022 Feb 28;77(1):367-373. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

25. Drobnis EZ, Nangia AK. Male Reproductive Functions Disrupted by Pharmacological Agents. Adv Exp Med Biol. 2017;1034:13-24. [PMC free article] [CrossRef] [Google Scholar]

26. Hosseinzadeh H, Sadeghnia HR, Ziaee T, Danaee A. Protective effect of aqueous saffron extract (Crocus sativus L.) and crocin, its active constituent, on renal ischemia-reperfusion-induced oxidative damage in rats. J Pharm Pharm Sci. 2005 Aug 22;8(3):387-93. [CrossRef] [Google Scholar]

27. Saeedi M, Rashidy-Pour A. Association between chronic stress and Alzheimer's disease: Therapeutic effects of Saffron. Biomed Pharmacother. 2021 Jan; 133:110995. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

28. Randez-Gil, F., Sánchez-Adriá, I. E., Estruch, F., & Prieto, J. A. (2020). The formation of hybrid complexes between isoenzymes of glyceraldehyde-3-phosphate dehydrogenase regulates its aggregation state, the glycolytic activity and sphingolipid status in Saccharomyces cerevisiae. Microbial Biotechnology, 13 (2), 562-571. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

29. Ghasemnejad-Berenji M. Immunomodulatory and anti-inflammatory potential of crocin in COVID-19 treatment. J Food Biochem. 2021 May;45(5):e13718. doi: 10.1111/jfbc.13718. Epub 2021 Apr 4. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

30. Anaeigoudari F, Anaeigoudari A, Kheirkhah-Vakilabad A. A review of therapeutic impacts of saffron (Crocus sativus L.) and its constituents. Physiol Rep. 2023 Aug;11 (15):e15785. [PubMed] [CrossRef] [Google Scholar]

31. Ni Y, Li L, Zhang W, Lu D, Zang C, Zhang D, et al.. Discovery and LC-MS characterization of new crocins in gardeniae fructus and their neuroprotective potential. J Agric Food Chem. (2017) 65:2936–46. 10.1021/acs.jafc.6b03866 [PubMed] [CrossRef] [Google Scholar]

32. Jalili C, Salahshoor MR, Pourmotabbed A, Moradi S, Roshankhah Sh, Darehdori AS, Motaghi M. The effects of aqueous extract of Boswellia Serrata on hippocampal region CA1 and learning deficit in kindled rats. Res Pharm Sci. 2014 Sep-Oct;9(5):351-8. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

33. Salahshoor MR, Khazaei M, Jalili C, Keivan M. Crocin Improves Damage Induced by Nicotine on A Number of Reproductive Parameters in Male Mice. Int J Fertil Steril. 2016 Apr-Jun;10(1):71-8. doi: 10.22074/ijfs.2016.4771. Epub 2016 Apr 5. [CrossRef] [Google Scholar]

34. Shokri, A., Baharara, J., & Amini, E. Evaluation the antioxidant effect of crocin on neonate Balb/C mouse spermatogonial stem cells. Cell and Tissue Journal (2016), 7(3), 219-229. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

35. Fani M, Mohammadipour A, Ebrahimzadeh-Bideskan A. The effect of crocin on testicular tissue and sperm parameters of mice offspring from mothers exposed to atrazine during

pregnancy and lactation periods: An experimental study. Int J Reprod Biomed. 2018 Aug;16(8):519-528. [CrossRef] [Google Scholar]

36. Baptissart M., Martinot E., Vega A., Sédes L., Rouaisnel B., de Haze A., Baron S., Schoonjans K., Caira F., Volle D.H. Bile Acid-FXRα Pathways Regulate Male Sexual Maturation in Mice. *Oncotarget.* 2016;7:19468–19482. [PMC free article] [PubMed] [CrossRef] [Google Scholar] [Ref list]

37. Sedes L., Desdoits-Lethimonier C., Rouaisnel B., Holota H., Thirouard L., Lesne L., Damon-Soubeyrand C., Martinot E., Saru J.-P., Mazaud-Guittot S., et al. Crosstalk between BPA and FXRalpha Signaling Pathways Lead to Alterations of Undifferentiated Germ Cell Homeostasis and Male Fertility Disorders. *Stem Cell Rep.* 2018;11:944–958. [PMC free article] [PubMed] [CrossRef] [Google Scholar] [Ref list]

38. Stępniewska J, Dołęgowska B, Cecerska-Heryć E, Gołembiewska E, Malinowska-Jędraszczyk A, et al. The activity of antioxidant enzymes in blood platelets in different types of renal replacement therapy: a cross-sectional study. *Int Urol Nephrol.* 2016;48:593–599.[PubMed] [CrossRef] [Google Scholar] [Ref list]

39. Bartosz G. *Second face of oxygen. Free radicals in nature.* Warszawa: Publishing House of PWN; 2003. [Google Scholar] [Ref list]

40. Sehitoglu I, Tumkaya L, Kalkan Y, Bedir R, Cure MC, Zorba OU, Cure E, Yuce S. Biochemical and histopathological effects on the rat testis after exposure to electromagnetic field during fetal period. Arch Esp Urol. 2015 Jul-Aug;68(6):562 [CrossRef] [Google Scholar]

41. Coutton, C.; Vargas, A.S.; Amiri-Yekta, A.; Kherraf, Z.-E.; Mustapha, S.F.B.; Le Tanno, P.; Wambergue-Legrand, C.; Karaouzène, T.; Martinez, G.; Crouzy, S. Mutations in CFAP43 and CFAP44 cause male infertility and flagellum defects in Trypanosoma and human. Nat. Commun. 2018, 9, 1–18. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

42. Shuling L, Sie Kuei ML, Saffari SE, Jiayun Z, Yeun TT, Leng JPW, Viardot-Foucault V, Nadarajah S, Chan JKY, Hao TH. Do men with normal testosterone-oestradiol ratios benefit from letrozole for the treatment of male infertility? Reprod Biomed Online. 2019 Jan;38(1):39-45. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

43. Luo L, Fang K, Dan X, Gu M. Crocin ameliorates hepatic steatosis through activation of AMPK signaling in db/db mice. Lipids Health Dis. 2019 Jan 8;18(1):11. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

44. Thirouard L, Holota H, Monrose M, Garcia M, De Haze A, Saru JP, Caira F, Beaudoin C, Volle DH. Analysis of the Reversible Impact of the Chemodrug Busulfan on Mouse Testes. Cells. 2021 Sep 13;10(9):2403. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

45. Aydos K, Aydos OS. Sperm Selection Procedures for Optimizing the Outcome of ICSI in Patients with NOA. J Clin Med. 2021 Jun 18;10(12):2687. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

46. Dcunha R, Hussein RS, Ananda H, Kumari S, Adiga SK, Kannan N, Zhao Y, Kalthur G. Current Insights and Latest Updates in Sperm Motility and Associated Applications in Assisted Reproduction. Reprod Sci. 2022 Jan;29(1):7-25. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

47. Gregoriou O, Bakas P, Grigoriadis C, Creatsa M, Hassiakos D, Creatsas G. Changes in hormonal profile and seminal parameters with use of aromatase inhibitors in management of infertile men with low testosterone to estradiol ratios. Fertil Steril. 2012 Jul;98(1):48-51. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

48. Haeri P, Mohammadipour A, Heidari Z, Ebrahimzadeh-Bideskan A. Neuroprotective effect of crocin on substantia nigra in MPTP-induced Parkinson's disease model of mice. Anat Sci Int. 2019 Jan;94(1):119-127. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

49. Tesarik J, Mendoza-Tesarik R. Cyclic Adenosine Monophosphate: A Central Player in Gamete Development and Fertilization, and Possible Target for Infertility Therapies. Int J Mol Sci. 2022 Dec 1;23(23):15068. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

50. Salehi E, Shadboorestan A, Mohammadi-Bardbori A, Mousavi A, Kargar-Abargouei E, Sarkoohi P, Omidi M. Effect of crocin and quercetin supplementation in cryopreservation medium on post-thaw human sperm quality. Cell Tissue Bank. 2024 Jun;25(2):531-540. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

51. Yaribeygi H, Maleki M, Rashid-Farrokhi F, Abdullahi PR, Hemmati MA, Jamialahmadi T, Sahebkar A. Modulating effects of crocin on lipids and lipoproteins: Mechanisms and potential benefits. Heliyon. 2024 Apr 3;10 (7):e28837. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

52. Ghasemnejad-Berenji M. Immunomodulatory and anti-inflammatory potential of crocin in COVID-19 treatment. J Food Biochem. 2021 May;45(5):e13718. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

53. Mesbahzadeh B, Hassanzadeh-Taheri M, Aliparast MS, Baniasadi P, Hosseini M. The protective effect of Crocin on cisplatin-induced testicular impairment in rats. Bmc Urology. 2021;21 (1):1–9. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

54. Mehdipour, M., Daghigh Kia, H., Najafi, A., Mohammadi, H., & Álvarez-Rodriguez, M. (2020). Effect of crocin and naringenin supplementation in cryopreservation medium on post-thaw rooster sperm quality and expression of apoptosis associated genes. Plos one, 15 (10), e0241105.

55. Sapanidou, V., Taitzoglou, I., Tsakmakidis, I., Kourtzelis, I., Fletouris, D., Theodoridis, A., ... & Tsantarliotou, M. (2015). Antioxidant effect of crocin on bovine sperm quality and in vitro fertilization. Theriogenology, 84 (8), 1273-1282. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

56. Salahshoor MR, Azari P, Roshankhah S, Zokaei A, Foroughinia A. The effect of Crocin on sperm parameters disorders induced by peripheral administration of nitrosamines in

male rats. J Med Life. 2022 Mar;15 (3):362-367. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

57. Rueangchainikhom W, Saeng-anan U, Vutyavanich T, Piromlertamorn W, Sanmee U. Effects of crocin on human sperm viability, motility, morphology, DNA fragmentation and reactive oxygen species levels after freezing and thawing. Thai Journal of Obstetrics and Gynaecology. 2023 Mar 9:115-24. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

58. El-Sheikh MM, Aziz MM, Abdelrahman SSM, Mohmad MAEH. The protective effect of crocin against testicular toxicity induced by ionizing radiation via AKT/FOXO pathway. Environ Toxicol. 2023 Dec;38(12):2981-2992. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

59. Sapanidou V, Taitzoglou I, Tsakmakidis I, Kourtzelis I, Fletouris D, Theodoridis A, Zervos I, Tsantarliotou M. Antioxidant effect of crocin on bovine sperm quality and in vitro fertilization. Theriogenology. 2015 Nov;84(8):1273-82. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

60. Tyagi V, Scordo M, Yoon RS, Liporace FA, Greene LW. Revisiting the role of testosterone: Are we missing something? Rev Urol. 2017;19(1):16-24. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

61. Kohn, T.P.; Louis, M.R.; Pickett, S.M.; Lindgren, M.C.; Kohn, J.R.; Pastuszak, A.W.; Lipshultz, L.I. Age and duration of testosterone therapy predict time to return of sperm count after human chorionic gonadotropin therapy. Fertil. Steril. 2017, 107, 351–357.e1. [Google Scholar] [CrossRef]

62. Jin W, Zhang Y, Xue Y, Han X, Zhang X, Ma Z, Sun S, Chu X, Cheng J, Guan S, Li Z, Chu L. Crocin attenuates isoprenaline-induced myocardial fibrosis by targeting TLR4/NF-κB signaling: connecting oxidative stress, inflammation, and apoptosis. Naunyn Schmiedebergs Arch Pharmacol. 2020 Jan;393(1):13-23. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

63. Jalili C, Tabatabaei H, Kakaberiei S, Roshankhah S, Salahshoor MR. Protective Role of Crocin Against Nicotine-induced Damages on Male Mice Liver. Int J Prev Med. 2015 Sep 10;6:92. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

64. Longobardi V, Zullo G, Cotticelli A, Salzano A, Albero G, Navas L, Rufrano D, Claps S, Neglia G. Crocin Improves the Quality of Cryopreserved Goat Semen in Different Breeds. Animals (Basel). 2020 Jun 26;10(6):1101. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

65. Wenzhi M, Lei A, Wu Z, Wang X, et al. Efficient and Safe Recipient Preparation for Transplantation of Mouse Spermatogonial Stem Cells: Pretreating Testes with Heat Shock1. Biol Rep. 2011; 85: 670-677. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

66. Olooto WE. Infertility in male; risk factors, causes and management- A review. JMicrobiol. Biotech. Res. 2012; 2(4): 641-645. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

67. Shabani S, Mashayekhi F, Shahangian SS, Salehi Z. Genetic polymorphism of glial cell-derived neurotrophic factor (GDNF) in male infertility. Br J Biomed Sci. 2019 Apr;76(2):86-88. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

68. Chen LY, Willis WD, Eddy EM. Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci U S A. 2016 Feb 16;113(7):1829-34. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

69. Khan GS, Tahir MZ, Zahoor MY, Rahman HU, Riaz A. Ameliorative effect of crocin on post-thaw quality, fertility-associated gene expression and fertilization potential of buffalo (Bubalus bubalis) bull sperm. Reprod Domest Anim. 2024 Jan;59(1):e14519. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

70. Garcia TX, Parekh P, Gandhi P, Sinha K & Hofmann MC 2017 The NOTCH ligand JAG1 regulates GDNF expression in Sertoli cells. Stem Cells and Development 26 585–598. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

71. Rahbar M, Asadpour R, Azami M, Mazaheri Z, Hamali H. Improving the process of spermatogenesis in azoospermic mice using spermatogonial stem cells co-cultured with epididymosomes in three-dimensional culture system. Life Sci. 2022 Dec 1;310:121057. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

72. Markouli, M., & Michala, L. (2023). Fertility potential in 5α-reductase type 2 deficient males. Journal of Pediatric Urology, 19 (1), 108-114. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

73. Wei R, Zhong S, Qiao L, Guo M, Shao M, Wang S, Jiang B, Yang Y and Gu C (2020) Steroid 5α-Reductase Type I Induces Cell Viability and Migration via Nuclear Factor-κB/Vascular Endothelial Growth Factor Signaling Pathway in Colorectal Cancer. Front. Oncol. 10:1501. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

74. Bonardi B, Massarotti C, Bruzzone M, Goldrat O, Mangili G, Anserini P, et al. Efficacy and Safety of Controlled Ovarian Stimulation With or Without Letrozole Co-administration for Fertility Preservation: A Systematic Review and Meta-Analysis. Front Oncol (2020) 10:574669. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

75. Mejia RB, Summers KM, Kresowik JD, Van Voorhis BJ. A Randomized Controlled Trial of Combination Letrozole and Clomiphene Citrate or Letrozole Alone for Ovulation Induction in Women With Polycystic Ovary Syndrome. Fertil Steril (2019) 111:571–578.e571. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

76. Mokhtarian, R., Rajabi, S., Zahedian, S., Jafarinejad-Farsangi, S., Hadizadeh, M., & Sadeghinejad, M. (2024, February). L'effet du safran et de ses extraits sur le traitement du cancer du sein: une revue narrative. In Annales Pharmaceutiques Françaises. Elsevier Masson. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

77. Cavallini G, Biagiotti G, Bolzon E. Multivariate analysis to predict letrozole efficacy in improving sperm count of non-obstructive azoospermic and cryptozoospermic patients: a pilot study. Asian J Androl. 2013; 15 (6):806. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

78. Haeri P, Mohammadipour A, Heidari Z, EbrahimzadehBideskan A. Neuroprotective effect of crocin on substantia nigra in MPTP-induced Parkinson's disease model of mice. Anat Sci Int. 2018;94:119-127. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

79. Salahshoor MR, Khazaei M, Jalili C, Keivan M. Crocin improves damage induced by nicotine on a number of reproductive parameters in male mice. Int J Fertil Steril 2016; 10: 71- 8. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

80. Suh KS, Chon S, Jung WW, Choi EM. Crocin attenuates methylglyoxal-induced osteoclast dysfunction by regulating glyoxalase, oxidative stress, and mitochondrial function. Food Chem Toxicol. 2019; 124: 367-73. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

81. Sefidgar SM, Ahmadi-hamedani M, Javan AJ, Sani RN, Vayghan AJ. Effect of crocin on biochemical parameters, oxidative/antioxidative profiles, sperm characteristics and testicular histopathology in streptozotocin-induced diabetic rats. Avicenna J Phytomed. 2019; 9(4): 347-55. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

82. Asadi MH, Zafari F, Sarveazad A, Abbasi M, Safa M, Koruji M, et al. Saffron improves epididymal sperm parameters in rats exposed to cadmium. Nephrourol Mon. 2014;6:e12125. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

83. Requena A, Herrero J, Landeras J, Navarro E, Neyro JL, Salvador C, Tur R, Callejo J, Checa MA, Farré M, Espinós JJ, Fábregues F, Graña-Barcia M; Reproductive Endocrinology Interest Group of Spanish Society of Fertility. Use of letrozole in assisted reproduction: a systematic review and meta-analysis. Hum Reprod Update. 2008 Nov-Dec;14(6):571-82. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

84. Pilutin A, Rzeszotek S, Wilk A, Klimaszewska K, Łukasiewicz J, Mafuta RL, Nagendran T, Ndambara R, Wiszniewska B. Effects of Letrozole Treatment and Vitamin C Supplementation on Morphology, Endoplasmic Reticulum Stress, Programmed Cell Death, and Oxidative Stress in the Small Intestine of Adult Male Rats. *Current Issues in Molecular Biology*. 2024; 46(3):1943-1954. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

85. Asuncion, M., Calvo, M. E., San Millán, J. L., Escobar-Morreale, H. F., & Garcia–Pascual, C. (2000). "A prospective randomised study of letrozole versus clomiphene citrate in the treatment of ovulatory dysfunction in polycystic ovary syndrome." Human Reproduction. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

86. Lin J, Wu F, Zhang K, Zhu Y, Wang B, Zhu Q and Lin J (2023) Impact of 2.5 mg versus 5 mg letrozole co-treatment in an antagonist protocol for IVF: a retrospective study. Front. Endocrinol. 14:1289595. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

87. Peivandi S, Jafarpour H, Abbaspour M, Ebadi A. Effect of letrozole on spermogram parameters and hormonal profile in infertile men: A clinical trial study. Endocr Regul. 2019 Oct 1;53 (4):231-236. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

88. Pan Y, Zhao X, Wang Y, Tan J, Chen D-X. Metabolomics integrated with transcriptomics reveals the distribution of iridoid and crocin metabolic flux in Gardenia jasminoides Ellis.

PLoS ONE. (2021) 16:e0256802. 10.1371/journal.pone.0256802 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

89. Hosen, M.B.; Islam, M.R.; Begum, F.; Kabir, Y.; Howlader, M.Z. Oxidative stress induced sperm DNA damage, a possible reason for male infertility. Iran J. Reprod. Med. 2015, 13, 525–532. [Google Scholar] [PubMed]

90. González-Marín, C.; Gosálvez, J.; Roy, R. Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. Int. J. Mol. Sci. 2012, 13, 14026–14052. [Google Scholar] [CrossRef] [PubMed]

91. Rashki Ghaleno, L.; Alizadeh, A.; Drevet, J.R.; Shahverdi, A.; Valojerdi, M.R. Oxidation of Sperm DNA and Male Infertility. Antioxidants 2021, 10, 97. [Google Scholar] [CrossRef]

92. Nowicka-Bauer, K.; Nixon, B. Molecular Changes Induced by Oxidative Stress that Impair Human Sperm Motility. Antioxidants 2020, 9, 134. [Google Scholar] [CrossRef]

93. Piomboni, P.; Focarelli, R.; Stendardi, A.; Ferramosca, A.; Zara, V. The role of mitochondria in energy production for human sperm motility. Int. J. Androl. 2012, 35, 109–124. [Google Scholar] [CrossRef]

94. Kaltsas A. Oxidative Stress and Male Infertility: The Protective Role of Antioxidants. *Medicina*. 2023; 59(10):1769. [Google Scholar] [CrossRef] [PubMed]

95. Bakhtiary Z, Shahrooz R, Ahmadi A, Zarei L. Evaluation of antioxidant effects of crocin on sperm quality in cyclophosphamide treated adult mice. Vet Res Forum. 2014 Summer;5(3):213-8. [Google Scholar] [CrossRef] [PubMed]

96. Nayan, M.; Punjani, N.; Grober, E.; Lo, K.; Jarvi, K. The use of assisted reproductive technology before male factor infertility evaluation. Transl. Androl. Urol. 2018, 7, 678–685. [Google Scholar] [CrossRef] [PubMed]

97. Ahmadi, S.; Bashiri, R.; Ghadiri-Anari, A.; Nadjarzadeh, A. Antioxidant supplements and semen parameters: An evidence based review. Int. J. Reprod. Biomed. 2016, 14, 729–736. [Google Scholar] [CrossRef] [PubMed]

98. Patel, A.S.; Leong, J.Y.; Ramos, L.; Ramasamy, R. Testosterone Is a Contraceptive and Should Not Be Used in Men Who Desire Fertility. World J. Mens Health 2019, 37, 45–54. [Google Scholar] [CrossRef]

99. Illiano, E.; Trama, F.; Zucchi, A.; Iannitti, R.G.; Fioretti, B.; Costantini, E. Resveratrol-Based Multivitamin Supplement Increases Sperm Concentration and Motility in Idiopathic Male Infertility: A Pilot Clinical Study. J. Clin. Med. 2020, 9, 4017. [Google Scholar] [CrossRef] [PubMed]

## Acknowledgments:

We thank the Department of Cancer Institute of Imam Khomeini Hospital (RA) and Islamic Azad University of Parand Branch in the implementation of this project.

**Authors' contribution**

**Conceptualization:** Shahrzad Nokhbeh Zaeem, Mitra Heydari Nasrabadi.

**Data curation:** Shahrzad Nokhbeh Zaeem.

**Formal analysis:** Shahrzad Nokhbeh Zaeem.

**Funding acquisition:** Shahrzad Nokhbeh Zaeem, Mitra Heydari Nasrabadi.

**Investigation:** Shahrzad Nokhbeh Zaeem.

**Methodology:** Shahrzad Nokhbeh Zaeem, Mitra Heydari Nasrabadi.

**Project administration:** Mitra Heydari Nasrabadi.

**Resources:** Mitra Heydari Nasrabadi.

**Software:** Shahrzad Nokhbeh Zaeem.

**Supervision:** Mitra Heydari Nasrabadi, Masoud Salehipour, Somayeh Ehtsham.

**Validation:** Shahrzad Nokhbeh Zaeem.

**Visualization:** Shahrzad Nokhbeh Zaeem.

**Writing–original draft:** Shahrzad Nokhbeh Zaeem.

**Writing–review & editing**: Mitra Heydari Nasrabadi.

## Funding sources:

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

## Ethical approval

The data included in this study were within the framework of routine clinical activity. All the study was carried out with the formal approval of the Institutional Review Board of our clinic and following the principles of the Declaration of Helsinki.

## Consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 1.xlsx