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Research Paper

Krocina, Improves in Vitro Fertilization Outcomes in Patients With Diminished Ovarian Reserve: A Randomized Controlled Trial

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ABSTRACT

Background: Diminished ovarian reserve (DOR) is a clinical syndrome with reproductive and endocrine disorders. This study aimed to examine the effect of crocin on oxidative stress, gene expression, oocyte maturation, and embryo quality in DOR patients who underwent a controlled ovarian hyperstimulation (COH) cycle.

Methods: As a clinical trial, this study involved 34 DOR patients trying to conceive by assisted reproductive technique who were divided into two groups (17 each): An intervention group receiving crocin (15 mg, once daily, for 12 weeks) and a control group receiving a placebo (tablets with the same form of the drug). Pre- and post-intervention, demographic information was gathered, and hormonal levels (follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2)) were measured. In the subsequent COH cycle, oocyte maturation, embryo quality, level of both superoxide dismutase (SOD) and reactive oxygen species (ROS) in follicular fluid, expression of *GDF9*, *BMP15*, and *Nrf2* genes in granulosa cells were measured.

Results: The collected data as a comparison between groups showed alteration of criteria in the intervention group as follows: Significant reduction of FSH ($P<0.01$), increased level of SOD in the follicular fluid ($P<0.0001$), decreased level of oxidative stress in the granulosa cells ($P<0.0001$), increased expression of *Nrf2* gene ($P<0.08$), and of *GDF9*, *BMP15* genes ($P<0.0001$) in the granulosa cells. The rate of oocyte maturation and embryo quality were significantly higher in the intervention group compared to the control group ($P<0.05$ and $P<0.005$, respectively).

Conclusion: Our study discussed how the Krocina supplement can slow down the progression of the disease by reducing the level of FSH, and oxidative stress, increasing the maturation rate of oocytes, and increasing the quality of embryos in women with DOR. Further research is needed to investigate the effect of crocin in improving fecundity for women with DOR.

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Introduction

Diminished ovarian reserve (DOR) is a pathological condition defined as a qualitative and quantitative reduction of oocytes in the ovarian cortex before age 40, which is experienced by more than 26% of young patients who have infertility. DOR, as an infertility-causing factor, is hallmarked by decreased anti-Müllerian hormone (AMH) levels and increased follicle-stimulating hormone (FSH) and FSH/luteinizing hormone (LH) ratio [1, 2]. DOR can turn into premature ovarian failure (POF) for 1 to 6 years. Hence, with no intervention, it can disrupt women's reproductive life [1]. Genetics, autoimmune disorders, chemotherapy, radiation therapy, lifestyle, and smoking can all contribute to DOR development [3]. The complex etiology of DOR includes a decrease in estrogen level, an increase in gonadotropin level and granulosa cell apoptosis, an improper nutritional and survival environment of oocytes with atresia of follicles, and a decrease in ovarian reserve [4, 5].

The typical treatment in DOR patients is hormone replacement therapy (HRT), including E2 valerate, progesterone capsules, clomid, and phenomycin. HRT, by reducing the serum level of FSH and increasing estradiol (E2), induces ovulation with monthly regular menstrual bleeding in patients. Long-term HRT may lead to an increased risk of ovarian cancer, ovarian hyperstimulation syndrome, breast cancer, and cardiovascular diseases such as venous thromboembolism and stroke [1, 2]. Owing to the abovementioned, there is a great demand to develop complementary or alternative treatments for DOR patients. Natural and herbal remedies are utilized due to fewer side effects and availability.

The spice saffron contains over 150 aromatic profiles, among which crocin is the main plant pigment that comes from the *Crocus sativus* plant, which has significant antioxidant and redox signaling roles based on numerous pharmacological research to have proven its antioxidant, anti-lipid, anti-inflammatory, and anti-cancer benefits [6, 7]. Crocin may reduce pituitary-hypothalamus sensitivity to testosterone and, subsequently, the negative feedback control of LH secretion. Moreover, crocin can play a role in the growth of anterior pituitary basophilic cells responsible for LH and FSH production [8].

The current study aims to investigate the effect of crocin on the hormonal levels (FSH, LH, and E2), oocyte maturation and embryo quality, follicular fluid superoxide dismutase (SOD), reactive oxygen species (ROS) level, and expression of *GDF9*, *BMP15*, and *Nrf2* genes in granulosa cells in the subsequent controlled ovarian hyperstimulation (COH) cycle of DOR patients.

Materials and Methods

Trial design

This study was conducted as a unicenter randomized, double-blind, placebo-controlled clinical trial. The aims and method of the study were fully explained to the patient, and according to the principles of the Declaration of Helsinki, informed written consent was obtained from all individuals. A total of 50 DOR patients aged less than 40 years were included in the study and randomly allocated to treatment and control groups in 1:1 from May 2021 to February 2022 in the in vitro fertilisation (IVF) and Infertility Center of Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran City, Iran.

Inclusion and exclusion criteria

The inclusion criteria were all DOR patients less than 40 years, which is defined as follows: (i) a woman with any risk factors for POR and or (ii) an abnormal ovarian reserve test (i.e. antral follicular count (AFC) <5–7 follicles or AMH <0.5–1.1 ng/mL) [9].

The exclusion criteria were uterine anatomical abnormalities, history of untreated endocrine problems, cardiovascular diseases, lung and liver disorders, severe and uncontrolled underlying diseases, unilateral or bilateral hydrosalpinx, prohibition of gonadotropin, oocyte donor patients, severe male infertility (azoospermia, oligoasthenoteratospermia), stage 4 endometriosis, patients with very low or high body mass index (BMI in kg/m^2) <18 or 30> respectively and the patient's request to leave the study.

Sample size

Due to the lack of effective randomized, blinded, controlled clinical trials that measure the effect of consumption of crocin on in vitro fertilization outcome, we performed this research as a pilot study. Thus, 50 DOR available patients were selected to participate in our study. Sixteen patients were excluded from the study due to not meeting the inclusion criteria, immigration, COVID-19 virus infection, and simultaneous consumption of antioxidants (Figure 1).

Randomization and masking

The internet-based central randomization system was applied in this trial, and 50 participants were randomly divided into two treatment and control groups based

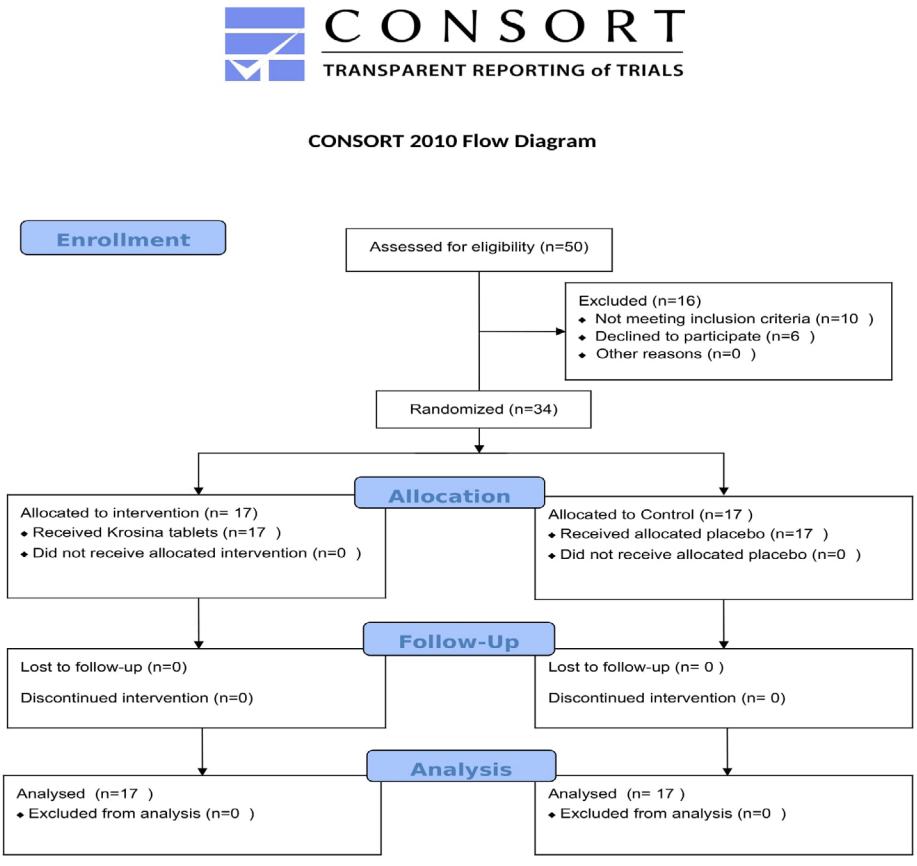

Figure 1. Flow diagram of the trial

on the computer-generated code in a 1:1 ratio. Using randomization codes, all investigators and participants were blinded to the study. The blinding code refers to the randomization methodology and its specific settings. Researchers not involved in outcome evaluation and statistical analysis rigorously adhered to this code.

Interventions

The intervention group received Krocina tablets (containing 15 mg of active ingredient crocin once a day) for 12 weeks, and the control group received placebo tablets with the same form of the drug. The crocin and placebo tablets were placed in numbered bags and provided to the participants based on a random list.

Baseline hormonal measurement and antral follicle count

The blood samples of the patients were collected immediately before and 12 weeks after consuming crocin and a placebo on the third day of the menstrual cycle. Blood serum was separated with centrifugation at 4000 rpm for 1 minute. Based on the manufacturer's instructions, FSH, LH, and AMH levels were measured using hormone assay kits. The number of antral follicles was recorded by transvaginal ultrasound in both ovaries, measuring all visible follicles ≥3 mm in diameter. Cystic morphologies larger than 12 mm were excluded.

COH cycle

The gonadotropin-releasing hormone (GnRH) antagonist protocol was performed for all patients. Daily subcutaneous injection of recombinant FSH (Cinnal F®, follitropin alfa, 300 U/d) started on the second day of the menstrual cycle and continued until the day of human chorionic gonadotropin (hCG) administration. Daily subcutaneous injection of dose 0.25 "ganirelix" (as a GnRH antagonist) was administered from day 6 of stimulation until the day of hCG administration. Subcutaneous highly purified human menopausal gonadotropin (hp-HMG) (PD-Homog®) daily dose of 300 IU started from day 8 of stimulation until the day of hCG administration. The cycle was canceled without ultrasound-visible follicles with a minimum size ≥11 mm on days 8 to 10 of stimulation. Also, 10000 IU hCG was administered when the size of the two follicles reached ≥18 mm as the final oocyte maturation indicator. About 34-36 hours later, oocyte retrieval and intracytoplasmic sperm injec-

Table 1. Designed primers for qRT-PCR

Gene Name	Primer Sequence
h-BMP15-f	CTGGCATGGCACTTCATCCT
h-BMP15-r	AGGCCCGCTATGTTTTGAGG
h-GDF9-f	GGCACGTACACATGACGGTCT
h-GDF9-r	CGCAGAGGTCAGGAAACTGTC
h-GAPDH-f	CTTTGGTATCGTGGAAGGAC
h-GAPDH-r	GCAGGGATGATGTTCTGG

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tion (ICSI) procedures were performed. Progesterone was started on the day of oocyte retrieval. A mature oocyte was a metaphase II (MII) with a visible first polar body in the cytoplasm. About 17-18 hours after ICSI, fertilization was confirmed as the appearance of two pronuclei (2PN). Immature oocyte rates were defined by cycle as the number of MI oocytes by the total number of retrieved oocytes (MI/oocyte) and as the number of germinal vesicles (GV) oocytes by the total number of retrieved oocytes (GV/oocyte). Third-day embryos were graded based on the number of blastomeres and the degree of fragmentation. Cleavage-stage embryos with normal growth on the third day contain 6 to 10 cells. Grade A and B embryos (based on Gardner's book) [10] were considered high-quality embryos. The fertilization rate was defined as the percentage of 2PNs per number of mature eggs injected/inseminated. Blastocyst rate was defined as the percentage of blastocysts per 2PNs.

Collection of follicular fluid and granulosa cells

Cumulus-oocyte-complex and follicular fluid were obtained by ultrasound-guided transvaginal oocyte retrieval 34–36 h after hCG injection. A pool of granulosa cells and follicular fluid was gathered from punctured follicles in each patient. The collected follicular fluid was centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to a 1.5-mL microtube and was stored at -80 ˚C. A needle cut the cumulus cells in each COC, and the granulosa cells were separated from the oocyte by enzymatic digestion and mechanical dissection, respectively. The collected granulosa cells were transferred to HTF (human tubal fluid) medium (supplemented with 10% albumin) and centrifuged at 1500 rpm for 5 minutes. The cell sediment was transferred to a 1.5-mL microtube and mixed with 90% fetal bovine serum (FBS)/10% dimethyl sulfoxide (DMSO) for freezing in liquid nitrogen for further flow cytometry analysis.

SOD assay

The SOD level in the collected follicular fluids was measured by the spectrophotometric method using the SOD kit (ZB-SOD, Germany). ZellBio GmbH assay kit uses the superoxide anion for conversion to hydrogen peroxide and oxygen under enzymatic reaction conditions. Finally, the product made a chromogen to a color, measured by colorimetry at 420 nm. The analysis of the results was calculated according to the Equation 1.

1.

$$SOD\ activity(U/mL)(V_p-V_c)›(V_p) \times 60$$

$$V_p=OD_{smaple\ 2min}-OD_{black\ 2\ min}\ V_c=OD_{smaple\ 2min}-OD_{black\ 2\ min}$$

$$e.g. V_p=0.0759\ and\ V_c=0.0183$$

$$SOD\ activity(U/mL)=(0.0759-(0.0183)›(0.0759)\times 60-45.52$$

ROS measurement method with flow cytometry

The ROS assay kit is a cell-based assay designed for measuring ROS activity within a cell using the cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). Once DCF-DA has diffused into cells, it is deacetylated by cellular esterase and converted to a non-fluorescent compound. ROS rapidly oxidizes into DCF, which was detected by fluorescent cytometry. The resulting fluorescence intensity is proportional to the ROS levels within the cell.

Measuring the expression level of *GDF9, BMP15, Nrf2* genes in the granulosa cells

The RNA was extracted from granulosa cells using the RNeasy mini kit (Qiagen, Germany) based on the manufacturer's protocol. The cDNA synthesis was performed

using a commercial kit (Easy cDNA synthesis kit, Parstous, Iran), and the synthesized cDNA was stored at -80 ˚C until further analysis. Allele ID software (version 7.5; Premier Biosoft International, USA) was used to design specific primers for *GDF9*, *BMP15*, and *Nrf2* genes and *GAPDH* as a housekeeping gene (Table 1). The StepOne RT-PCR system (Applied Biosystems, USA) was utilized to conduct the reactions. Each reaction consisted of RNase-free water, RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), and specific primers with or without cDNA. Each reaction was performed in triplicate.

Statistical analysis

The normality of data distribution was measured by the Shapiro-Wilk test. We used the independent and dependent sample t-tests to compare the studied groups. Experimental results are reported as Mean±SD, and statistical analysis is performed with Graph Pad Prism software, version 9. A value of P<0.05 is considered statistically significant.

Results

Demographic information and hormonal characteristics of the patients

Comparison of baseline parameters between groups in Table 2 showed the baseline parameters of two groups (placebo and crocin) pre-intervention. There were no significant differences between placebo and intervention groups regarding age, BMI, AFC, FSH, LH, E2, and AMH parameters.

Consumption of crocin after 12 weeks in the treated group resulted in a significant reduction of FSH level. Crocin also slightly increased LH levels but did not affect E2 and AMH levels. Also, 19% of participants had primary infertility, and 5% had secondary infertility (Table 3).

Effects of crocin on the COH and ICSI cycle outcomes

Oocyte maturation and high-quality embryo rates showed significant increases in the crocin group compared to placebo after 12 weeks (Table 4).

Effects of crocin on the level of SOD in the follicular fluid

Analysis of SOD level in the follicular fluid showed a significant increase in the crocin group compared to the placebo (P<0.0001) (Figure 2).

The effect of crocin on the level of oxidative stress in granulosa cells

Analysis of ROS levels in granulosa cells showed a significant decrease in the crocin group compared to placebo (P<0.0001) (Figure 3).

The effect of crocin on the mRNA level of *GDF9*, *BMP15*, and *Nrf2* genes in the granulosa cells

The *GDF9* and *BMP15* expression showed significantly elevated levels in the crocin group compared to the control (P<0.0001) (Figures 4a and 4b). The expression level of *Nrf2* was higher in the crocin group compared to the control group (P<0.08), though it was insignificant (Figure 4c).

Discussion

DOR etiology can be related to various factors, including autoimmune ovarian damage, infectious agents, toxins, genetic disorders, and environmental factors. Most DOR cases are idiopathic and have no identifiable cause. One of the probable causes of idiopathic DOR may be chronic inflammation by ecological toxins that destroy the antioxidant system [11]. The destruction of

Table 2. Baseline characteristics of the patients

Variables	Mean±SD		P
	Placebo (n=17)	Crocin (n=17)	
Age (y)	32±5	29±5	0.77
BMI (kg/m²)	22±3	23±3	0.45
AFC (n)	1.8±0.8	2.1±0.9	0.65
Infertility duration (y)	3.7±2.1	4.3±2.5	0.28

BMI: Body mass index; AFC: Antral follicular count.

Table 3. Hormonal characteristics of the intervention group

Variables	Mean±SD		P	Mean±SD		P	P
	Baseline Profile			After 12 Week Treatment			
	Placebo (n=17)	Crocin (n=17)		Placebo (n=17)	Crocin (n=17)		
FSH (IU/mL)	14.6±2.8[a]	13±3[a]	0.18	14.9±3[a]	10.4±3.5[b]	0.006	0.004
LH (IU/mL)	7.3±2.5[a]	5.8±1.6[a]	0.09	7.2±2.8[a]	5.5±1.7[a]	0.08	
E2 (pg/mL)	98.9±28.7	102±27.6	0.15	98±27.6	104±29.1	0.65	
AMH (ng/mL)	1±0.4[ab]	0.8±0.5[a]	0.48	1 ±0.4[ab]	1.3±0.8[b]	0.29	0.024

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Abbreviations: BMI: Body mass index; AFC: Antral follicle count; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; E2: Estradiol; AMH: Anti-Müllerian hormone.

Notes: Different letters indicate statistically significant differences at P<0.05.

the antioxidant system can lead to the accumulation of a large amount of ROS in the tissues, promoting oxidative stress and subsequent tissue dysfunction. Oxidative damage results in diseases related to severe ovarian dysfunction, such as polycystic ovary syndrome (PCOS), DOR, premature ovarian failure, and ovarian aging [12, 13]. Studies have shown that oxidative stress can cause a decrease in ovarian reserve by inducing telomere shortening, mitochondrial dysfunction, apoptosis, and inflammation [14]. Hence, antioxidant factors play an essential role. Nuclear factor erythroid 2 (Nrf2) is expressed in the cytoplasm of many cells and plays a critical role in maintaining the balance between antioxidant and oxidant systems [12]. Nrf2 presents in the cytoplasm of granulosa cells (GCs); its amount decreases with the reduction of the ovarian follicular reserve [15]. During oxidative stress, Nrf2 combines with the nucleus's antioxidant response element (ARE) and activates 500 downstream antioxidant genes [14]. One of the activated antioxidant enzymes is SOD. It is the main antioxidant

Table 4. COH and ICSI cycle outcomes

Variables	Mean±SD/%		P
	After 12 Weeks		
	Placebo (n=17)	Crocin (n=17)	
Retrieved oocyte (n)	2±1.04	2.25±1.2	0.89
Immature oocytes (n)	0.91±0.9	0.18±0.4	0.003
MII (n)	0.75±0.96	1.75±1.2	0.5
Embryo (n)	1±0.93	1.5±1.16	0.3
High-quality embryo (n)	0.18±0.4	1.07±0.86	0.005
Immaturity rate (%)	70	28	0.01
MII rate (%)	35.4	71.2	0.05
Fertilization rate (%)	41.6	63.8	0.05
Cleavage rate (%)	66.66	66.66	0.71
High-quality embryo rate (%)	66.6	88.6	0.04

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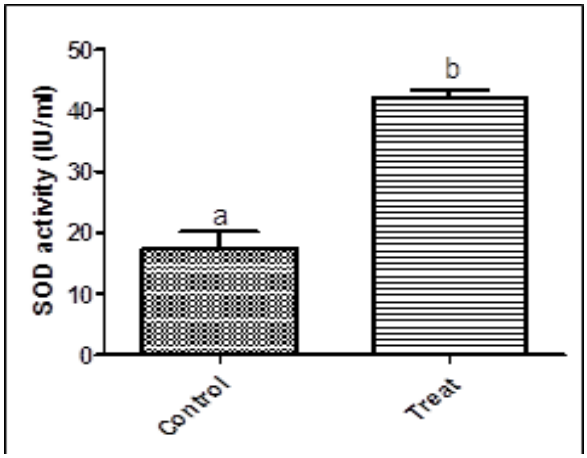

Figure 2. Levels of antioxidants in follicular fluid

Notes: Data are shown as Mean±SD, different letters indicate statistically significant differences at P<0.05.

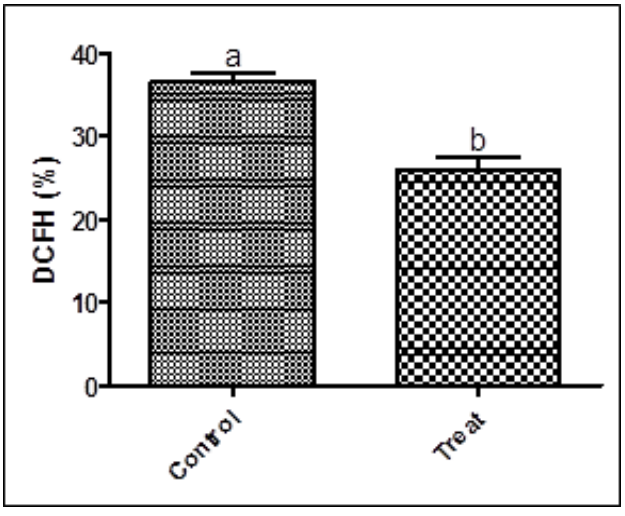

Figure 3. The level of oxidants in the granulosa cells statistically significant differences at P<0.05.

Notes: Different letters indicate statistically significant differences.

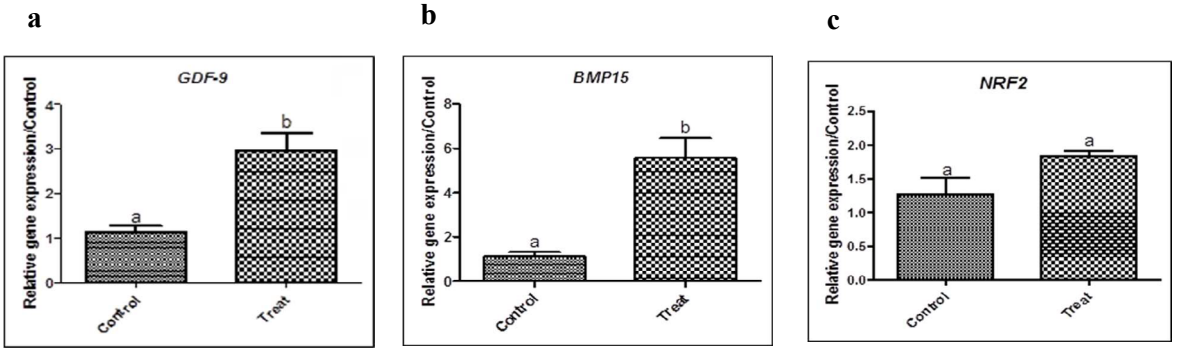

Figure 4. The gene expression level of *GDF9* (a), *BMP15* (b), and *Nrf2* (c)

Notes: Different letters indicate statistically significant differences at P<0.05. The control group received no treatment, the treatment group received Crocin.

defense against ROS. Clinical studies have shown that mRNA and protein levels of the SOD antioxidant in the GCs of older women or young women with DOR undergoing IVF are significantly lower than those of unaffected counterparts [16, 17].

Crocin is a well-known carotenoid chemical compound in traditional medicine. Various studies have shown its high anti-radical activity and anti-inflammatory, anti-apoptotic, and possibly anti-aging activity [18]. By decreasing the adverse effects of oxidative stress, supplementing the IVM medium with crocin has improved mouse oocyte's nuclear maturation rate and subsequent growth potential [19, 20]. Saffron and its compounds have been assumed to be a therapeutic and protective agent in various pathological conditions in tissues such as the liver, heart, pancreas, lung, and brain through the Nrf2 signaling pathway [21]. Antioxidant activity of crocin during porcine oocyte maturation in vitro could reduce the damage of oxidative stress by directly inhibiting the ROS and upregulating the expression of antioxidant genes and SOD levels [22]. We showed that consuming crocin for 12 weeks can increase the Nrf2 expression in the granulosa cells of DOR patients. The level of SOD enzyme in the follicular fluid increased in the DOR patients as well. Previous studies have proven that the expression of *GDF9* and *BMP15* genes is reduced in patients with DOR [23]. The expression of these two genes plays an essential role in the cross-talk between granulosa cells and oocytes [24] by suppressing the apoptosis of granulosa cells and increasing the effect of FSH on them. Since granulosa cells are essential for oocyte nutrition and maturation, any changes in the expression of genes can affect folliculogenesis and oocyte maturation [25]. Studies have shown that *GDF9* and *BMP15* work synergistically to influence the proliferation of GCs [26]. Furthermore, consuming crocin as a supplement for 12 weeks in DOR patients increased the expression of *GDF9* and *BMP15* genes in granulosa cells.

The effect of adding crocin to the maturation medium during pig IVM and IVF has been evaluated previously. The results of this study have shown that 400-500 µg/mL crocin supplementation during oocyte maturation can increase the maturation rate (86.8% vs 72.8%) and cleavage rate (62.50% vs 53.33%) compared to the control group. Therefore, crocin can increase the rate of oocyte maturation [22]. Along with previous laboratory studies, we showed that in vivo consumption of crocin for 12 weeks could increase the oocyte maturation rate in DOR patients. We have also shown that consuming crocin for 12 weeks in DOR patients could improve the quality of embryos, which has not been investigated in any study.

The ratio of FSH/LH is considered an essential index in evaluating ovarian reserve. This ratio is reduced in DOR individuals [12]. In previous studies, crocin has restored serum FSH, LH, and E2 levels to normal range in the rat model of PCOS [8]. Moreover, crocin may reduce the sensitivity of the pituitary-hypothalamus to testosterone and subsequently reduce the negative feedback on LH hormone secretion [8]. As a therapeutic effect of crocin, it can increase the growth of basophil cells in the anterior pituitary gland, which is responsible for the production of LH and FSH [27]. Crocin can secrete LH hormone by affecting the hypothalamus axis and increasing the amount of GnRH hormone secretion [28]. Here, crocin consumption for 12 weeks significantly increased the FSH level and the ratio of FSH to LH in DOR patients.

Conclusion

To sum up, it seems that crocin can fight DOR pathology by improving the ovarian microenvironment through improving hormonal balance, having antioxidant activity, and activating antioxidant pathways to an acceptable extent, and it can be suggested as a complementary treatment before starting IVF cycles.

Ethical Considerations

Compliance with ethical guidelines

The trial was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Code: IR.SBMU.MSP.REC.1402.61). In addition, this trial was registered in Iran's Clinical Trials Registry (IRCT) (Code: 20220516054872N1).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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