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Original Article

A comparative study of saffron aqueous extract and its active ingredient, crocin on the *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture of mouse oocytes

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ABSTRACT

Objective: Reactive oxygen species have effects on gamete quality and gamete interaction; they influence spermatozoa, oocytes, embryos, and their environment. In this study, we evaluated the antioxidant effect of different concentrations of saffron (*Crocus sativus* L.) aqueous extract (SAE) and its ingredient, crocin, on the improvement of *in vitro* maturation (IVM) and subsequent *in vitro* fertilization (IVF) and embryo development of mouse oocytes.
Materials and methods: Cumulus oocyte complexes were collected from ovaries, and germinal vesicle oocytes were cultured in the presence of SAE and crocin. SAE was added at dosages of 5 μg/mL, 10 μg/mL, and 40 μg/mL; dosages of crocin were 50 μg/mL, 100 μg/mL, and 400 μg/mL. All dosages were added to maturation medium and a group without SAE or crocin was considered as the control group. Following IVM, metaphase II oocytes were fertilized and cultured *in vitro* in order to observe embryo development.
Results: Both SAE and crocin improved the rate of IVM, IVF, and *in vitro* culture. Addition of 40 μg/mL SAE to maturation medium significantly increased the rate of IVM, IVF, and *in vitro* culture ($p < 0.05$). Furthermore 100 μg/mL crocin significantly increased the IVM rate compared to the control group ($p < 0.05$).
Conclusion: Use of SAE during IVM can affect on IVM, IVF, and early embryo development in a dose-dependent manner. SAE appears to have a stronger effect than pure crocin.

Introduction

New laboratory techniques are used in the infertility clinics and *in vitro* fertilization (IVF) is one of these assisted reproduction techniques. Preparation of oocytes before IVF is one of the important factors responsible for the developmental competence of embryos following IVF [1]. *In vitro*-matured oocytes have been used in some laboratories because their use makes it feasible to obtain a large number of oocytes from ovaries with lower cost. Studies indicate that *in vitro*-matured oocytes have lower developmental competence than *in vivo*-derived oocytes [2]. *In vitro* culture conditions have higher concentrations of O_2 than *in vivo* conditions. Oxygen tension in the oviduct, is approximately from one-quarter to one-third that of atmospheric tension [3]. Previous studies have demonstrated that higher levels of reactive oxygen species (ROS) produced through *in vitro* cultures can adversely affect many aspects of culture condition and reduces fertilization [4], embryo development, and pregnancy rates [5]. Oocytes maturation and development are lower in media when compared to media supplemented with chemicals such as amino acids or antioxidants [6,7]. Various antioxidant agents exist in cells and their functions are complementary in the cellular defense system [8]. Agarwal et al [9] reported that the addition of various antioxidants such as β-mercaptoethanol, taurine, hypotaurine, vitamin E, and vitamin C to the culture media increased the rate of blastocyst formation.

Since ancient times, saffron (*Crocus sativus* L.) has been used as a medicinal plant and a culinary spice [10,11]. It has been used as an

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eupeptic, antispasmodic, gingival sedative, anticatarrhal, nerve sedative, carminative, expectorant, diaphoretic, stimulant, stomachic, aphrodisiac, and emmenagog, and promotes the diffusion of oxygen in different tissues[11]. Anticonvulsant [12], antidepressant [13], anti-inflammatory [12], antitumor, and learning and memory improving effects [11,14–17] are other pharmaceutical properties of saffron. Most of those properties are related to its antioxidant activity [18]. In this study of the antioxidant properties of saffron extract and crocin, its active ingredient, we examined their effects on *in vitro* maturation (IVM), IVF, and *in vitro* culture (IVC) of mouse oocytes.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). All procedures performed on animals received the prior approval of the Ethics Board at Royan Institute, Tehran, Iran.

Plant materials

Saffron stigmas were collected from Ghaen (Khorasan Razavi Province, Northeast Iran). The plant was authenticated and voucher specimen coded 408 was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran.

Preparation of the plant extract

About 10 g of stigmas were ground to a powder and dried in the shade at ambient temperature. Dried stigmas were decocted in water for 30 minutes. Subsequently, the extract was filtered and concentrated using a rotary evaporator apparatus (Heidolph, Schwabach, Germany). The final weight of stigmas was 2 g. The SAE was maintained at 4°C throughout the experiments. Before adding SAE to maturation medium, it was filtered by 0.22 μm filters.

Animals

Animal experiments were carried out according to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW publication, National Institutes of Health, 80–23).

Ovaries were obtained from 6–8-week-old female Naval Medical Research Institute (NMRI) mice (purchased from Pasture Institute, Tehran, Iran). Animals were kept under controlled light and temperature with enough water and food. They were kept in a 12-hour light–dark condition.

Collection of germinal vesicle oocytes

Animals were killed by cervical dislocation and their ovaries were transferred into dissecting medium that contained minimum essential medium (MEM-α) supplemented with 5% fetal bovine serum, 100 IU penicillin, and 100 IU streptomycin. Cumulus oocyte complexes were retrieved from the follicles under a stereomicroscope (Olympus, Tokyo, Japan) using two 27-gauge needles.

IVM

IVM medium was MEM-α supplemented with 100 IU penicillin, 100 IU streptomycin, 5% fetal bovine serum, 7.5 IU/mL recombinant human follicular stimulating hormone (Organon, Oss, The Netherlands) and 100 IU/mL human chorionic gonadotrophin (Organon). Different concentrations of SAE (5 μg/mL, 10 μg/mL, and 40 μg/mL) or crocin (50 μg/mL, 100 μg/mL, and 400 μg/mL) were added to maturation medium. About 10–15 cumulus oocyte complexes were transferred to 25 μL drops, which were covered by mineral oil.

IVF

Epididymal sperm suspensions were prepared from adult male NMRI mice and incubated for 1 hour in the IVF medium to ensure sperm capacitation. IVF and capacitating medium were consisted of T6 medium supplemented with 25 mg/mL bovine serum albumin (equilibrated at 37°C in 5% CO_2). After IVM, metaphase II oocytes were washed in IVF media and three or four oocytes were transferred to 50 μL microdroplets, which were previously covered by mineral oil. For IVF, about 2×10^6 spermatozoa/mL were added to the droplets containing oocytes. Sperm and oocytes were incubated at 37°C with 5% CO_2 for 4–6 hours.

In vitro development

Inseminated oocytes were collected, washed, and transferred to 20 μL IVC droplets (KSOM with 4 mg/mL bovine serum albumin). During IVC (96 hours), the numbers of two-cell, four-cell, morula, and blastocyst embryos were recorded with inverted microscope (Nikon, Tokyo, Japan) daily.

Statistical analysis

The analysis of variance (one way ANOVA) and Duncan protected least-significant tests, using SAS version 1.9 (Raleigh, NC, USA), were applied for all statistical analysis. Some percentages of values were subjected to arc sine transformation before analysis. All data are expressed as mean ± standard error of the mean. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effect of saffron extract on in vitro maturation and early embryo development

As shown in Table 1 and Fig. 1, the addition of SAE to maturation medium increased the rate of oocyte maturation, two-cell embryo formation, and blastocyst formation. High dosage of the extract (40 μg/mL) was the most effective dosage. The maturation rate, and two-cell and blastocyst formation were significantly increased in this group compared to the control group ($p < 0.05$). There was no significant difference between the other groups and the control group.

Effect of crocin in oocyte in vitro maturation and early embryo development

As is evident in Table 1 and Fig. 1, all three dosages of crocin improved IVM rate and two-cell, four-cell, morula, and blastocyst formation. However, a significant increase in the IVM rate was seen only in the group treated with 100 μg/mL ($p < 0.05$). The rate of development was increased in all stages of embryo development when compared to the control group. However, there were no significant differences between the experimental groups and the control group.

Discussion

Obtaining good quality oocytes prior to IVF is one of the most important factors affecting developmental competence of subsequent embryos [1]. During IVC, a higher concentration of O_2 compared to *in vivo* conditions is one of the most important

Table 1
Percentage of *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture in different groups.[a]

Groups	IVM	24 h after IVF	48 h after IVF	72 h after IVF	96 h after IVF
	MII oocyte rate[b]	2-cell–4-cell[c]	4-cell–8-cell[c]	Morula–blastocyst[c]	Total blastocyst[c]
Control	53 ± 0.07	50 ± 0.09	26 ± 0.01	16 ± 0.07	11 ± 0.07
5 μg/mL SAE	73 ± 0.06	77 ± 0.06	49 ± 0.08	27 ± 0.08	21 ± 0.07
10 μg/mL SAE	76 ± 0.06	72 ± 0.07	40 ± 0.05	25 ± 0.08	22 ± 0.07
40 μg/mL SAE	79 ± 0.06*	78 ± 0.04*	56 ± 0.06	53 ± 0.05	38 ± 0.05*
50 μg/mL crocin	79 ± 0.07	70 ± 0.07	43 ± 0.03	32 ± 0.05	29 ± 0.07
100 μg/mL crocin	78 ± 0.04*	67 ± 0.05	52 ± 0.06	36 ± 0.02	29 ± 0.04
400 μg/mL crocin	75 ± 0.06	70 ± 0.09	44 ± 0.07	31 ± 0.06	24 ± 0.04

Data are presented as mean ± standard error of the mean.
*Comparison of groups with control group is significant ($p < 0.05$).
SAE = saffron aqueous extract.
[a] All experiments were repeated eight times.
[b] Percentage of metaphase II (MII) oocytes.
[c] Percentage of two-cell, four-cell, eight-cell, or morula and blastocyst embryos in relation to the MII oocytes.

challenges [3]. Different studies have demonstrated that higher levels of ROS, which are produced during *in vitro* cultures, adversely affect IVF [4,5], subsequent embryo development, and clinical pregnancy rates [5].

Recently, use of natural antioxidant agents in culture medium has been considerably investigated [19,20]. In the current research the effects of the SAE and crocin, which is one of its components, on IVM, IVF, and IVC were studied.

There are two types of cellular defense against free radical complications: enzymatic [catalase, superoxide dismutases, and glutathione (GSH) peroxidase] and nonenzymatic (glutathione, α-tocopherol, and vitamin C) free radical scavenging systems [21]. Several studies have indicated that saffron extract and its carotenoids have an effect on the activation of enzymes with antioxidant properties [22–24]. It has been proven that carotenoids of saffron such as crocins as an antioxidant agent effect on apoptosis signaling pathways and prevent morphological changes promoted by apoptosis and DNA fragmentation induced by tumor necrosis factor-α and serum-glucose deprivation. Studies on rat pheochromocytoma (PC-12) cells indicate that the antioxidant effects of saffron are related to increased GSH synthesis, which can protect those cells from death [25–28]. It has been demonstrated that GSH level is an indicator of cytoplasmic maturation in oocytes [29,30]. Furthermore it has been reported that sperm DNA condensation and male pronucleus formation was mediated by intracellular GSH levels in mature oocytes [31–33]. In the current research higher maturation rate and higher early embryonic development with the effect of saffron extract, indicated its positive effects during *in vitro* culture. As it was observed that higher dosages of SAE increased the percentage of oocyte maturation and embryonic development, it

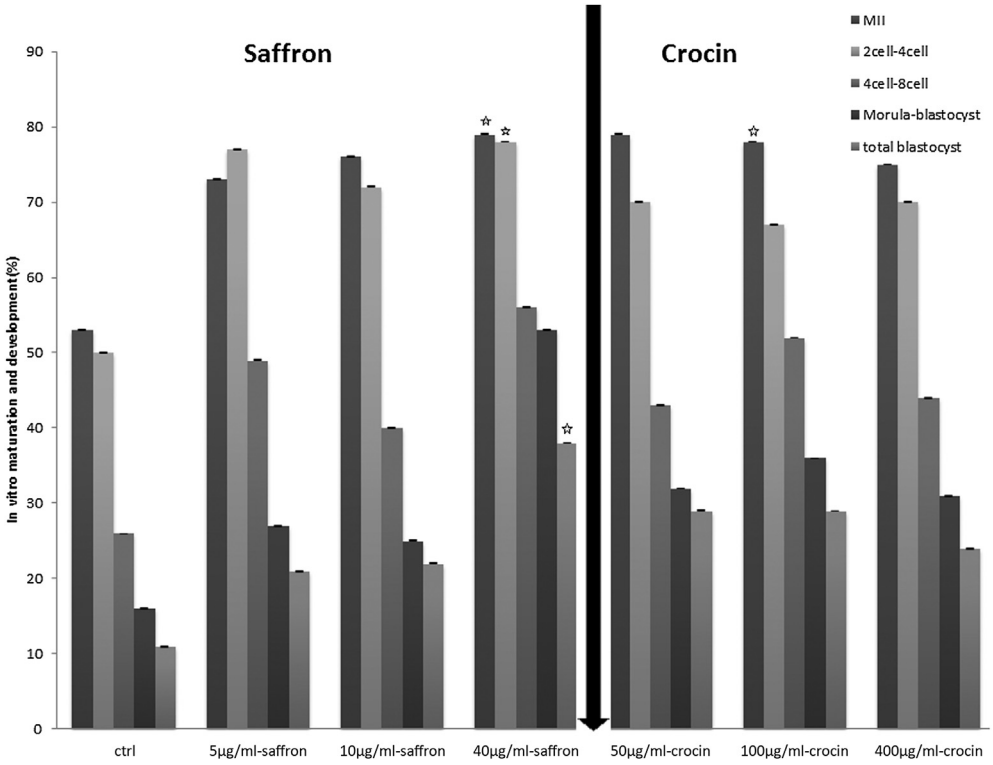

Fig. 1. *In vitro* maturation and *in vitro* development rate of mouse embryos. This demonstrates the improvement of oocyte maturation and embryo development rate for each group and percentage of metaphase II (MII) oocytes and embryo development expressed as mean ± standard error of the mean. All experiments were repeated eight times.

seems that the effect of saffron extract was dose dependent. Increasing effects of SAE on IVM rate are probably due to its antioxidant effect, which is related to the effect of its components on oocyte maturation. Saffron components such as crocins (water soluble carotenoids which are glycosyl esters of crocetin), crocetin, dimethyl crocetin, safranal, and flavonoids are free radicals scavengers [11,34]. Phenol and flavonoid compounds, which are widely found as secondary metabolites in plants, are important due to their ability to serve as antioxidants [35]. Flavonoids have been shown to be effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals [36,37].

As Yen et al [38] and Siddhuraju et al [39] reported, the ferric reducing power of bioactive compounds such as flavonoids was associated with antioxidant activity. According to Karimi et al [40], flavonoids appeared to reduce Fe^{3+}. This activity in saffron flavonoids is another observation that confirms its antioxidant properties [40]. Adverse effects of iron in the medium during *in vitro* culture have been discussed previously [41]. It has been shown that saffron stigma also contains anthocyanin pigments [42], which react with ROS and neutralize free radicals because of electron deficiency [43]. It has been shown that anthocyanins are free radical scavengers [44] with synthesis GSH that protect cells against free radicals. Anthocyanins stimulate activation of ɣ-glutamyl synthetics and elevate GSH levels in culture medium [45]. As mentioned earlier, increased GSH levels during oocyte maturation are associated with the improvement of subsequent embryo development [46].

Among the constituents of saffron stigmas, crocins and crocetin are the most abundant carotenoids to have their pharmacological properties investigated and antioxidant and antitumor effects established [11,14,47]. These carotenoids scavenge free radicals, especially superoxide anions, and therefore they may protect cells against oxidative stress [48] In the current research, oocyte maturation was increased when 100 μg/mL crocin was added to maturation medium. In the case of reproduction and gamete research, there has been a report demonstrating the antioxidant effect of crocin on sperm [49]. Our results indicate that despite 100 μg/mL crocin increasing the IVM rate, IVC rate of oocytes was not increased compared to the control group. Addition of the most effective dosage of SAE increased all *in vitro* procedures including IVM, IVF, and IVC rates of oocytes. Therefore it seems that the addition of SAE to maturation medium was more effective than its pure component crocin. Saffron contains proteins, sugars, vitamins (such as riboflavin, thiamin, and vitamins A and C), amino acids, and minerals in addition to flavonoids, anthocyanins, and crocins [11,34]. It seems that synergistic action of all those components with antioxidant properties in SAE is probably responsible for stronger antioxidant effect in comparison with its pure component, crocin. There has been a report about the increasing effect of a plant extract with antioxidant properties on IVM and *in vitro* development of mouse oocytes [19,20]. Similarly our results confirm that the addition of natural antioxidants such as SAE may be a promising way to reduce adverse effects of oxidative stress during IVM to increase IVM outcomes.

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