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The Analysis of the *Foxp2* and *ASCL1* Expression in Saffron-Treated Mice

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Abstract

Background & Objective: The aim of this study was to investigate the effect of saffron extract on the embryo development of mice.

Materials & Methods: Pregnant NMRI mice were randomized into control and treatment groups at 25, 50, and 100 mg/Kg saffron doses. Saffron extract was administered to mice by gavage on days 7-12 of pregnancy. On the 17th day of pregnancy, the embryos were removed from the uterus and their weight and height measured. Moreover, their brain tissue has been evaluated histologically. Subsequently, the expression of the *Foxp2* and *Ascl1* genes in the brain tissue was assessed using the real-time PCR method.

Results: All embryos were aborted in mothers that received 100 mg/Kg of saffron. At dose 50 mg/Kg, only embryos of a mother reached the end of pregnancy. Embryos treated with 25 mg/Kg saffron were significantly heavier than controls ($P<0.05$). Furthermore, the tail length was significantly shorter ($P<0.05$). Histological findings showed that there was no difference between the control and treated groups, and the brain tissue was well developed. *Foxp2* and *Ascl1* genes were significantly overexpressed in both the 25 and 50 mg/Kg treatment groups compared to the control group ($P<0.05$).

Conclusion: The results of this study showed that saffron extract can have significant effects in low concentrations (25 mg/kg) on the development of the mouse embryos as well as the expression of *Foxp2* and *Ascl1* genes.

Keywords: saffron, embryo, mouse, *Foxp2* gene, *Ascl1* gene

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Introduction

Crocus sativus L. is a flowering plant of the Iridaceae family and is commonly referred to as saffron. It is a perennial plant which is grown in several countries of moderate and dry climate such as Iran, India, Greece, Spain, Italy, Turkey, and China. Saffron is mainly used as a spice and food color and to a lesser degree as a textile dye or perfume (1). The presence of three main secondary metabolites give saffron therapeutic values; Crocin related to the red color characteristic, Picrocrocin related to the bitter taste characteristic, and Safranal (2,6,6-trimethyl-1, 3-cyclohexadiene-1-carboxaldehyde) related to the aromatic odor characteristic. Crocins are

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very useful in the food industry because they are able to create bright orange to yellow colors. Transcrustin ester (β-D-gentiobiosyl) is the most abundant ester with high water solubility whose solubility is mainly attributed to the carbohydrate parts. Approximately, 30% of the dry weight of the spice is composed of Crocin. Monoterpene aldehyde picrocrocin is responsible for the bitter taste of saffron. Safranal is a cyclic terpenic aldehyde produced from picrocrocin. It is the main component of *C. sativus* essential oil, appears to be the primary cause of the unique smell of saffron, and constitutes about 60% of the volatile compounds of saffron (2).

According to the findings of the available reports, the toxicity of saffron is relatively low and completely dependent on the dose (3). In animal studies, the LD50 for oral consumption of saffron was determined at 20.7 g/Kg (4). Studies on the harmful effects of saffron have given conflicting results. In some studies, it has been demonstrated that the injection of 1.2-2 g/Kg saffron may cause nausea, vomiting, diarrhea, and bleeding (5), whereas in other studies, even up to 4 grams daily for several days showed no toxicity even in pregnant women (6). However, the above studies were carried out in Germany and it is not clear whether common saffron was used or whether wild saffron native to Germany was tested. Consumption of over 10 grams of saffron may cause side effects such as loss of appetite, insomnia, nausea, vomiting and confusion (5).

Saffron has been shown to have teratogenic effects and its use can lead to abortion. Occasionally, this substance is also used to interrupt an unwanted pregnancy. Reports indicate that consumption of heavy doses of saffron can cause miscarriage by stimulating uterine contractions. Reports also exist on the harmful effects of saffron on fetal health. Considering the effects of saffron on different organs and its common use as a food additive, its effects need to be carefully considered and an effective dose should be determined for the health of the fetus (7). In the present study, Iranian saffron extract was administered orally at doses of 25, 50, and 100 mg/Kg to pregnant mice, and its effects on fetal growth and development were evaluated through macroscopic and histological assessment. *FOXP2* was the first clearly identified gene related to speech and language development. The initial discovery was obtained through the studies of a large intergenerational family (KE family) with mainly hereditary speech and language development disorders (8). Also, mutation analysis in embryonic brain and manipulation of *Ascl1* activity in neural stem cell cultures have shown that *Ascl1* is indeed required for normal proliferation of neural progenitors (9). Considering the important role of these genes in the development of the fetus and especially the development of the brain, their expression was investigated in the saffron-treated mice.

Materials and Methods

A hundred grams of saffron (Sahrakhiz-Iran) were finely powdered with mortar and soaked in double distilled water for an hour to prepare an aqueous extract. Subsequently, it was homogenized and centrifuged for 10 minutes at 2000 rpm until the particles were deposited and the supernatant solution was retained for the experiments. The dose was calculated based on the weight of dried saffron used to prepare 1 ml of extract (10).

Animals' treatment

In this study, 24 NMRI female mice were used under standard conditions and in accordance with ethical Principles of Laboratory Animal Care. In all experiments, 10-week-old female mice weighing 29-32g were used to ensure that they were mature. Mice were kept in the lab for a week before experiments to adjust to the environment. In this experimental study, to induce pregnancy, one male mouse was placed in a cage for every two female mice and they were examined every morning to observe the vaginal plaque. The day of plaque observation

was considered to be day zero of pregnancy. After confirming pregnancy by observing the vaginal plaque, the animals were randomly divided into four groups (n=6) under standard conditions, including the untreated control group and three experimental groups. The experimental groups were administered 25, 50 and 100 mg/Kg by gavage on days 7 to 12 of pregnancy.

Macroscopic studies

On the 17th day of pregnancy, the mice were euthanized using 40 mg/Kg of xylazine and 400 mg/Kg of ketamine by the intrapritoneal injection and the embryos were removed from the uterus. Crown-rump length (CRL), head length, tail length and length of fore and hind limbs were measured by calipers and the weight of embryos was measured with the digital scale.

Histological studies

After macroscopic study, the brains of the embryos were separated. Some brains were fixed in formalin (10%), and some were frozen in liquid nitrogen. After 48 h of fixation, brain tissues were sectioned (5 µm) using a microtome and stained by H & E method. Sections of forebrain, midbrain, hind brain and cerebellum were prepared and studied by the light microscopy (Zeiss, Germany) (11).

Molecular studies

Investigating the effect of saffron extract on the level of transcription and expression of *Foxp2* and *Ascl1* genes in the embryos whose mothers were treated with saffron was done using Real time PCR method. To this end, RNA extraction of the fetal brain tissue was carried out using the Total RNA Kit (GeneAll RiboEx, Korea) in accordance with the manufacturer's instructions. Quantitative and qualitative evaluation of extracted RNA was performed using spectroscopy (Thermo Scientific™ NanoDrop 2000, USA) and electrophoresis on 1% agarose gel, respectively. The cDNA synthesis was carried out with the Yekta Tajhiz Azma Kit. Finally, the Real time PCR was performed using the Master Mix 2x SYBR Green qPCR kit (Yekta Tajhiz Azma, Iran). The designed primers are listed in Table 1. The temperature profile includes a denaturation cycle for 3 minutes at 95˚C, 45 amplification cycles including 5 seconds at 95°C and 30 seconds at 60˚C, and finally a melting cycle at 50-99˚C. Fold change calculation was done through the formula $2^{-\Delta\Delta Ct}$ using the *GAPDH* as a reference gene.

Table1. Sequence of primers used in Real time PCR

Genes	Primers	Sequences	Product length (bp)
Foxp2	Forward	GTAATCACCCCAGCCAGTGT	173
	Reverse	GACTCCATGATAGCCTGCCTT	
Ascl1	Forward	TCCCCCTTTGATCGTGCTTC	130
	Reverse	CGGCTCCACTCTCCATCTTG	
GAPDH	Forward	GAAGCTTGTCATCAACGGGA	180
	Reverse	GAAGGGGCGGAGATGATGAC	

Data Analysis

Data analysis was carried out using the SPSS v.22 software and the T-test method. The p-value < 0.05 was considered statistically significant

Results

Macroscopic studies

The mean number of embryos in control groups was 13, which is within the normal range (Figure 1A). The control embryos were within the normal range in terms of weight, and morphology (Figure 1D). In mothers treated with 100 mg/Kg saffron extract, hyperemic and cystic ovaries, undeveloped embryos and absorbed embryos were found. Consequently, there were no embryos available in this group (Figure 1B). At dose 50 mg/Kg only one mother reached the end of pregnancy and 6 fetuses were obtained (Figure 1E). At dose 25 mg/Kg, there were two pregnant mothers each of which had two embryos and the remaining embryos were either absorbed or did not develop (Figure 1C and 1F).

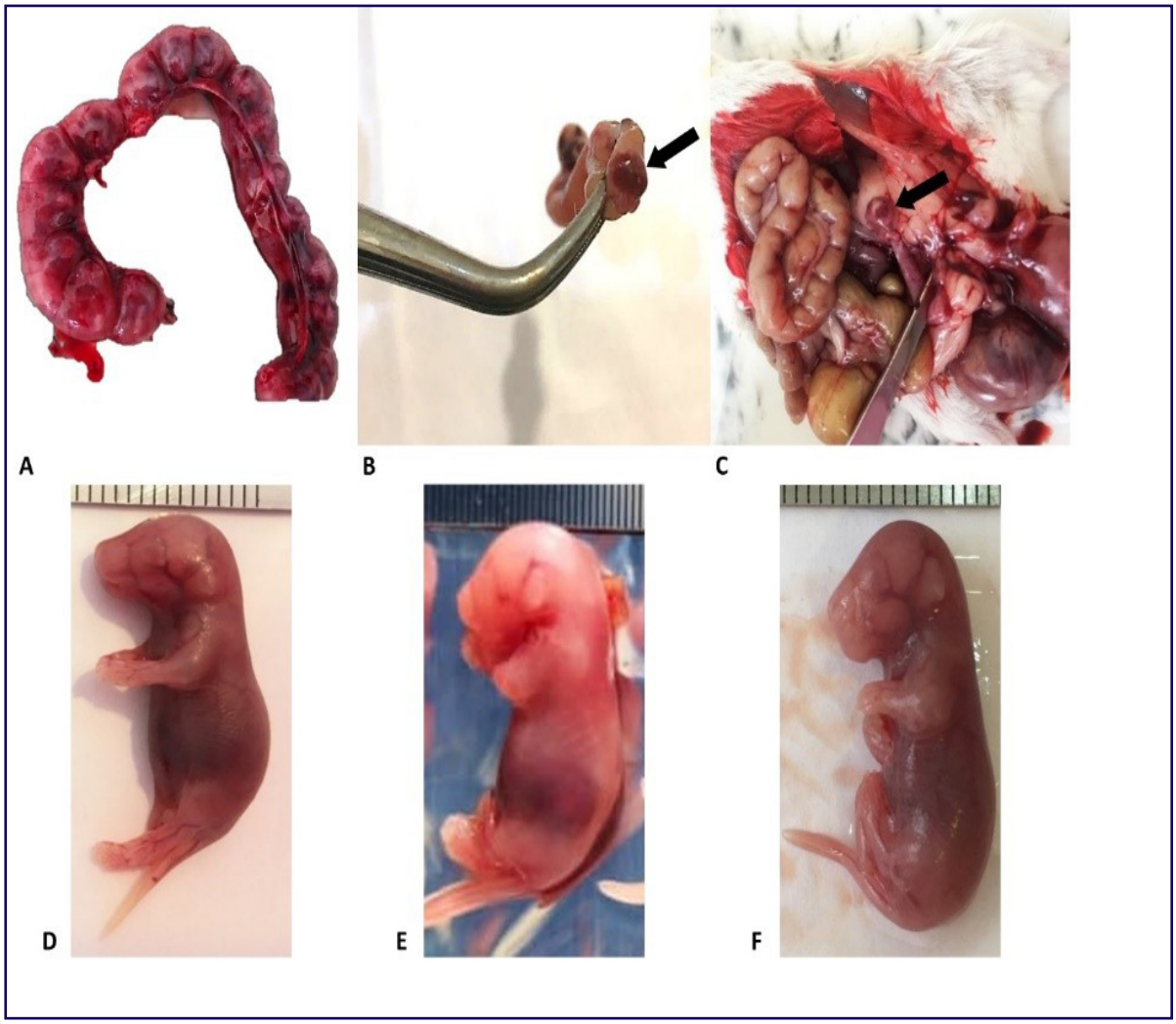


Figure1. Macroscopic investigation of ovaries and embryos in the studied groups (A: ovaries of the control group, B: hyperemic ovaries and no formation of embryos in the 100 mg/Kg treated group, C: ovaries in the 25 mg/Kg treated group, D: control group, E:50 mg/Kg group and F: 25 mg/Kg group)

Comparison of developmental parameters

Developmental parameters of embryos including weight, CRL, length of head, fore and hind limb and tail in embryos of control group and group treated with 25 and 50 mg/Kg of saffron extract were measured and compared (Table 2). The findings showed that the weight of 25 mg/Kg saffron-treated embryos was significantly higher than that of the control group. At the same time, the tail length was significantly lower than that of the control group. Other examined factors did not reveal any differences between the two groups. The 50 mg/Kg treated group did not show any significant difference with the control group.

Table2. The Average of developmental parameters in groups treated with saffron extract and control group

Groups	Number of embryos/ pregnant mother	Weight (g)	CR length (mm)	Head length (mm)	Hand length (mm)	Foot length (mm)	Tail length (mm)
Control	13.37	0.92 ± 0.12	21.59 ± 1.44	7.32±0.56	8.17±1.47	9.08±1.67	11.53±1.08
25 mg/Kg	2	1.06 ± 0.02*	20.37±0.26	6.99±0.44	6.96±0.2	8.22±0.11	9.95±0.17*
50 mg/Kg	6	0.92	19.56	7.55	7.93	8.92	11.94
100 mg/Kg	0	-	-	-	-	-	-

*Analyzed by independent t-test

Histologic studies

Cross-sections of the brain tissue of embryos treated with 25 and 50 mg/Kg of saffron extract and embryos in the control group were prepared and compared. Sections were prepared from the forebrain, midbrain, hindbrain and cerebellum. The results indicated that there was no histological difference in these two groups and that brain tissue was well formed in both groups (Figure 2). At the same time, the cerebral cortex is well stratified. The distinct layers, from superficial to deep, include the marginal zone, cortical plate, subcortical plate, intermediate zone, ventricular and subventricular zone (SVZ and VZ). The cortical plaque is continuous, but it shrinks considerably from the neocortex to the growing hippocampus and creates a layer of pyramid cells in these areas. Currently, morphological differences between the different Cornu Ammonis 1 (CA1), CA2 and CA3 regions are not apparent. As cells continue to proliferate and differentiate in the surrounding areas, especially in the

anterior part, the ventricular system becomes fainter. The two halves of the thalamus join at the midline to form the interthalamic junction. The pituitary gland was clearly differentiated. The adenohypophysis, neurohypophysis, and the narrow median zone that separates them were all clearly discernible in H&E-stained sections. The pineal gland is well developed.

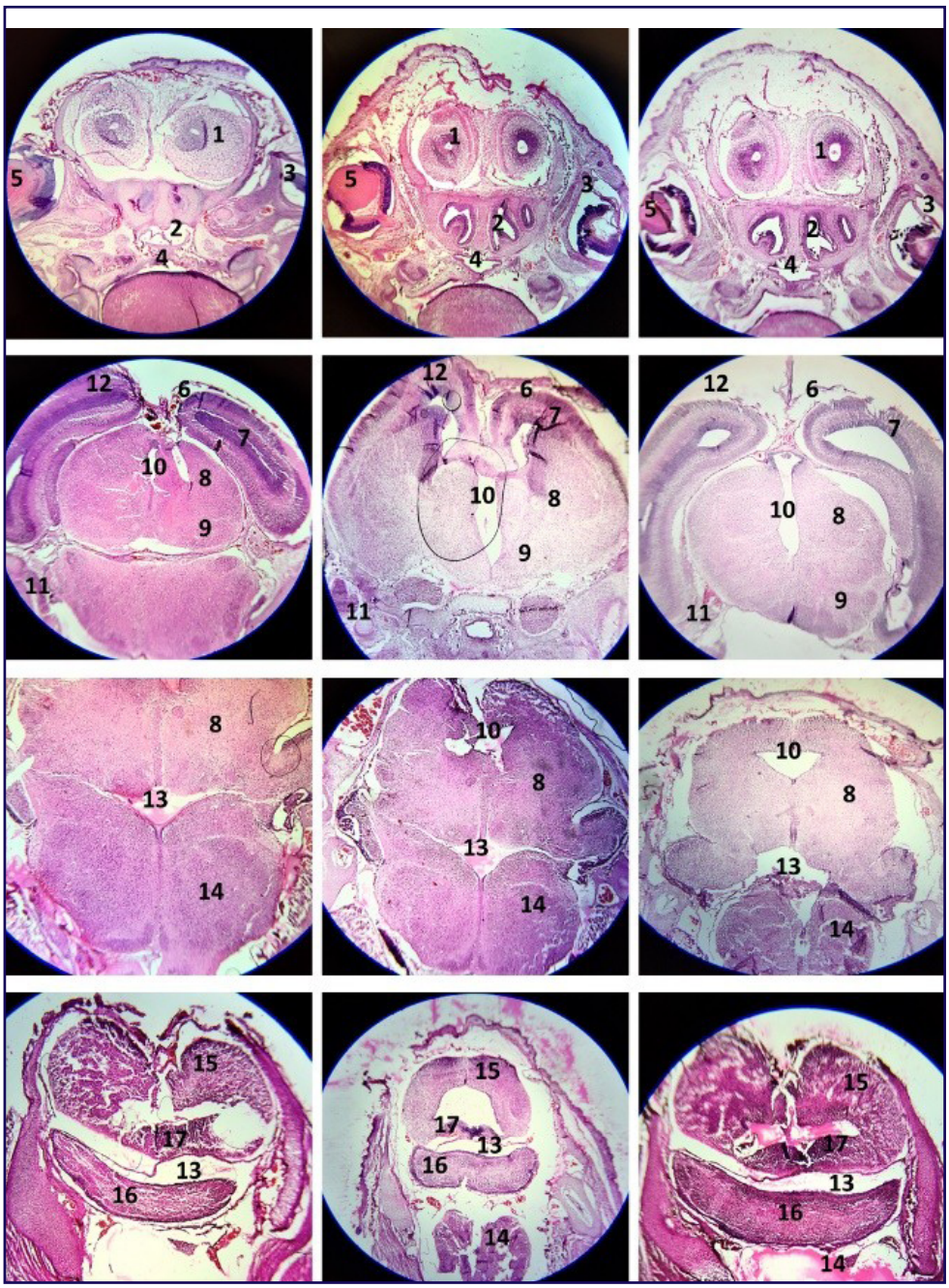


Figure2. Cross-sections of embryo brain. First row: forebrain, second row: midbrain, third row: hindbrain, fourth row: cerebellum, left column: control group, middle column: 25 mg/Kg treated group, right column: 50 mg/Kg treated group (1: olfactory lobe, 2: nasal cavity 3: optic vesicle, 4: nasopharyngeal canal, 5: lens, 6: telencephalon, 7: hippocampus, 8: thalamus, 9: hypothalamus, 10: third ventricle, 11: olfactory lobe, 12: lateral ventricle, 13: Fourth ventricle, 14: Quadruple ridges, 15: Medulla, 16: Cerebral pons, 17: Cerebellum)

Molecular studies

Expression of the *Foxp2* gene in the brain of control and treated embryos at doses of 25 and 50 mg/Kg saffron extract was compared. The findings showed that this gene was significantly overexpressed in both treatment groups compared to the control group (Chart 1A). There was no significant difference in the *Foxp2* expression between the two treatment groups. Expression results of *Ascl1* gene showed a significant upregulation of *Ascl1* in both groups treated with saffron extract relative to the control group. Furthermore, the highest level of expression was observed in the 50 mg/Kg group, which was much higher than the 25 mg/kg group (Chart 1B).

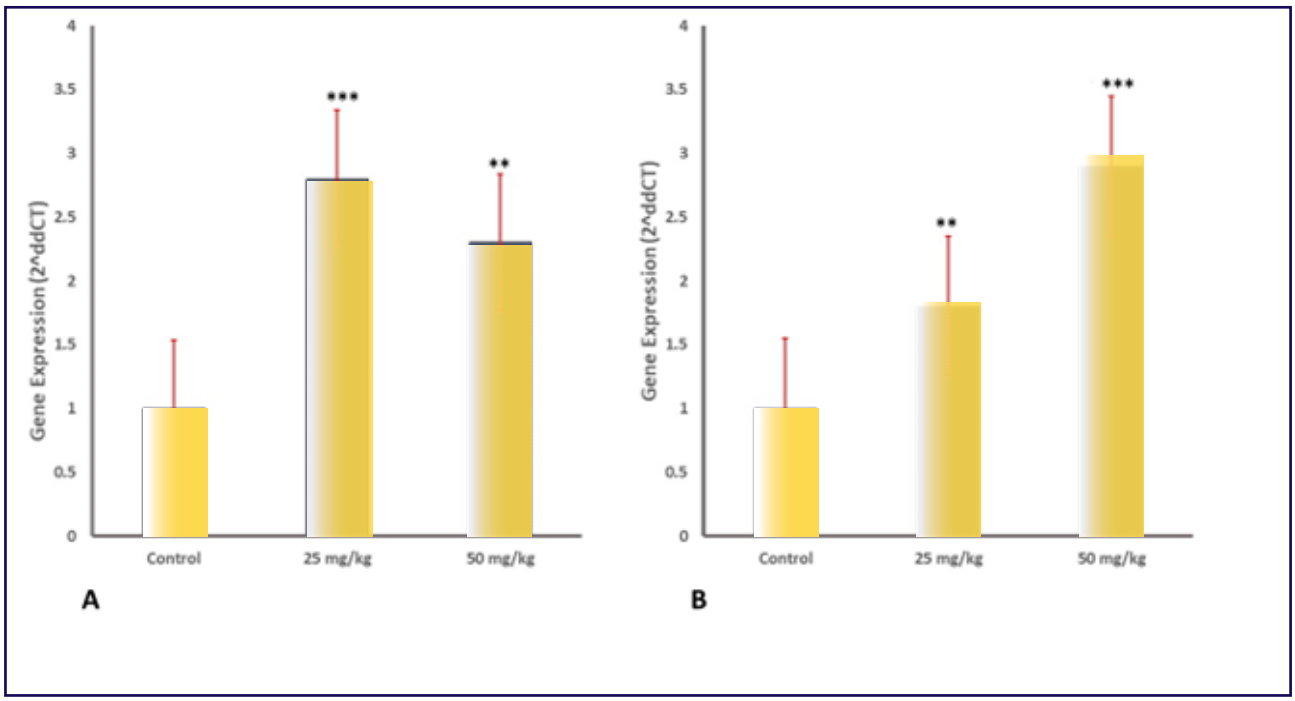


Chart 1. Comparison of *Foxp2* (A) and *Ascl1* (B) gene expression in the brain of control and embryos treated with doses of 25 and 50 mg/Kg of saffron extract and analyzed by independent t-test, (**P<0.01, *** P<0.001)

Discussion

The results of the embryological studies showed that in the mothers who were treated with a dose of 100 mg/Kg of saffron, all the embryos were aborted. At dose 50 mg/kg, only the fetuses of one mouse reached the end of pregnancy. The weight of embryos treated at 25 mg/Kg saffron was significantly higher than control group. In addition, the tail length of the experimental group embryos was significantly less than control group. Other parameters examined showed no difference in both groups. The results of the histological examinations showed that there was no difference in the control group and the treatment group with the dose of 25 and 50 mg/Kg, and the brain tissue was well formed. Also, in the molecular studies, it was shown that *Foxp2* and *Ascl1* genes significantly overexpressed in both the 25 and 50 mg/Kg dose treatment groups compared to the control group.

In an experimental study, Tavana et al. investigated the effects of aqueous saffron extract on in vitro maturation, fertilization and embryo development of mouse oocytes. Cumulus oocyte

complexes (COCs) were collected from ovaries of NMRI female mice aged 6-8 weeks and cultured in an IVM medium containing 0, 5, 10, 20 and 40 µg/mL aqueous extract of saffron. The results indicated that maturation was significantly higher in all groups treated with different concentrations of aqueous saffron extract than in the control group. Also, lower concentrations of saffron aqueous extract (5 and 10 µg/mL) increased in vitro oocyte fertilization rates and growth ability compared to the control group (12). In the present study, it was similarly shown that low concentrations of saffron extract (25 mg/Kg) had positive effects on the growth of mouse embryos, while teratogenic effects of saffron extract were observed with increasing concentration.

Groszer et al. (2008) reported that motor learning is reduced in mice heterozygous for a previously identified mutation in the KE family (13). Also, Chabout et al. (2016) stated that these mice produce shorter sequences of ultrasonic sounds with less complexity compared to wild type littermates (14). French et al. (2019) showed that with homozygous knockout, *Foxp2* expression in the cerebral cortex, striatum, and cerebellum of mice modulates various aspects of motor behavior (15). However, Urbanus et al. (2020) showed that selective gene deletion in any of these brain regions did not significantly alter the production of ultrasonic sounds (16). It is interesting to note that while some studies have indicated that the selective deletion of *Foxp2* in the cerebral cortex of mice does not affect the development of cortical structures during embryogenesis (17, 18), mice with *Foxp2* knockout have been reported in the cerebral cortex to show altered social behaviors (17, 19). Database research indicates that the expression of *FOXP2* under the influence of saffron has not yet been studied. Peresent results showed that both studied concentrations (25 and 50 mg/Kg) were capable of enhancing the expression of the *FOXP2* gene relative to the control group that may indicate the positive role of 25 and 50 mg/Kg doses of saffron in fetal brain development.

Proneural genes such as *Ascl1* are known to enhance cell cycle arrest and neuronal differentiation when expressed in neural progenitor cells. The mechanisms through which proneural genes activate neurogenesis, particularly the genes that they regulate, are often unknown. Castro et al. (2011) performed a genome-wide characterization of *Ascl1* transcriptional targets in the embryonic brain and in neural stem cell cultures by analyzing the location and expression profiles of embryos overexpressing or mutant for *Ascl1*. A wide range of molecular and cellular functions indicate that *Ascl1* directly controls the specification of neuronal progenitors and the subsequent stages of neural differentiation and growth. *Ascl1* also regulates the expression of a large number of genes involved in cell cycle progression, including the regulators of the canonical cell cycle and oncogene transcription factors. Castro and Vasconcelos (2014) reported that *Ascl1* expression in a variety of precursors is restricted along the rostro-caudal axis of the developing brain and spinal cord (20). In several gain-of-function studies, the role of *Ascl1* in the development of neural differentiation and characteristics in embryos has been supported (21, 22). In addition, Nakada et al. (2004) showed that the overexpression of *Ascl1* in the chick spinal cord leads to the arrest of progenitors from cell cycle, migration and subsequent expression of specific proneural and neuronal markers (23). On the contrary, the studies of Casarosa et al. (1999) showed that *Ascl1* downregulation in mice leads to reduced neural progenitors in embryos (24).

In the present study, the effect of two concentrations of 25 and 50 mg/Kg of saffron extract on the expression of *Ascl1* in the brain of mice was investigated, and the results showed that both concentrations significantly increased the expression of the *Ascl1* gene in the brains of mice embryos compared to the control group. The 50 mg/Kg concentration was also demonstrated to be more effective than 25 mg/Kg in inducing expression of the *Ascl1* gene. These results show that saffron is likely to induce

growth and development in the fetal brain.

Conclusion

Our results showed different and dose-dependent effects of saffron in line with other studies. The concentration of 100 mg/Kg resulted in no embryo formation. Moreover, the concentrations of 25 and 50 mg/Kg caused a decrease in the number of fetuses. In the histological section, by studying the brain tissue, it was found that in the treated groups, similar to the control group, brain development was done well. Also, the expression of *FOXP2* and *Ascl1* genes involved in brain development was evaluated and the results showed that both genes significantly increased in both the 25 and 50 mg/Kg treatment groups compared to the control group. Due to the important role of the concentration in the observed effects, it is necessary to determine the effect of each concentration of saffron extract by conducting detailed and comprehensive tests.

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Conflict of interest

The authors declare no conflict of interest.

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