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Original Article

Alteration of Neurodevelopmental Gene Expression Following Prenatal Exposure to Aquatic *Crocus Sativus* L. Extract in Mice

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Abstract

Background: *Crocus sativus* (saffron) is used since ancient times as a medicine and spice. Various studies have demonstrated its antioxidant, anticancer, and anti-inflammation properties. In folkloric Iranian medicine, saffron is known as an abortifacient agent and some investigations announced it as a teratogen. The current study aimed to investigate the *C. sativus* extract effect on the fetal brain.

Methods: The NMRI female mice were randomly divided into the control group and three saffron aquatic extract treated groups (25, 50, and 100 mg/kg of saffron aquatic extract). The fetuses were treated during 7-12 days post coitum (dpc). The fetuses were morphologically evaluated. Fetal brain tissues were investigated by histology and real-time PCR for *Foxg1*, *Foxa2*, *Wif1*, and *Fgf8* expression.

Results: We found that three treatments reduced the number of fetuses. Fetuses of 25 mg/kg treated were significantly heavier ($P<0.001$) and had shorter tails ($P<0.001$) than controls. No difference was observed among treated and control groups in histological prospect. *Foxa2* and *Wif1* expressions dose-dependently increased ($P<0.0001$), while *Foxg1* mRNA level increased in 25 mg/kg treatment ($P<0.0001$). *Fgf8* expression decreased significantly in 25 mg/kg and 50 mg/kg treatments ($P<0.0001$ and $P<0.001$, respectively).

Conclusion: These findings suggested that although no difference was observed in the histology of the fetal brain, the alteration of mentioned genes could affect the cellular biochemistry, molecular structures, and cell types in the developing brain.

Keywords: Saffron, Neurogenesis, Mouse, Fetus, *Foxg1*, *Foxa2*, *Wif1*, *Fgf8*.

1. Introduction

Normal development of the fetus is an important issue during pregnancy. Prenatal exposure to chemical teratogens could result in a divergence of this important process [1]. Many of these kinds of chemicals are able to pass the placental barrier and reach the fetus [2].

These chemicals may not be toxic to the mother; however, directly interfere

the fetal development. The adverse effects of chemicals on the fetus may lead to malformation, developmental delay, functional alteration, or even fetal death [3]. Herbal products are widely used during pregnancy around the world. Because of the general belief in their safety; however, some undesirable results have been reported from the consumption [4,5].

Saffron is one of the most precious spices so-called "red gold". It is obtained from the dried stigma of *Crocus sativus* L. flower. It has been cultivated in Asian and Mediterranean countries since ancient times. Saffron is an acknowledged spice because of its aroma, flavor, and dye [6]. Primarily saffron was considered a valuable herbal medicine. In traditional medicine, saffron is consumed to heal depression, menstruation disorders, bronchospasms, and liver disease [7]. Nowadays researches have revealed that saffron has anti-depression, antitumor, antioxidant, and anti-inflammatory properties. *C. sativus* contains components responsible for its pharmacological properties. Several studies examined the beneficial effect of saffron on central nervous system disorders, coronary heart disease, respiratory diseases, immunological disorders, and reproductive problems [8-11]. Despite some findings about the protective effects of saffron on fetus development, few studies and Iranian traditional medicine questioned these results. Two studies on the effects of saffron and its constituents on maternal pregnancy situation and fetuses showed the raise in preterm delivery and a reduced number of newborns [12,13]. In addition, the altered weight of newborns and malformation in fetuses could be caused by saffron consumption during pregnancy [12-15]. The book "Canon" implies the role of saffron in the induction of abortion [16]. In Iranian ethnomedicine, the dose of saffron to induce miscarriage is more than 10 grams which could result in some adverse side effects [7]. Our knowledge about saffron effects on the fetus, particularly on the developing fetal nervous system, is vague. Therefore, in this study, we tried to analyze the expression of four genes playing crucial roles in brain development.

In the developing brain, the signaling centers (organizers) are responsible for the secretion of signaling molecules to mediate regional patterning as well as neuronal subtype specification. In some signaling centers, the fibroblast growth factor 8 (Fgf8), sonic hedgehog (Shh), and wingless/integrated proteins (Wnts) are merged to orchestrate the correct specification [17-19].

Fgf8 simultaneously patterns the development of the cortex, midbrain, hindbrain, and diencephalon [17]. In addition, it is documented that Fgf8 is a crucial factor in the differentiation of dopaminergic neurons [20]. Shh signaling pathway controls brain patterning, size, shape, and cells' destination. Shh synergistically regulates brain development with many factors, including Wnt [21]. Wnt signaling plays several important roles in development, such as early patterning and cell fate determination. Therefore, Wnt signaling is a target of dynamic regulation in a tempo-spatial way via various modulators to guarantee normal neurogenesis [22]. Wnt inhibitory protein (Wif1) (as an antagonist of Wnt in the early steps of the pathway) interrupts Wnt signaling pathway [23]. The *Wif1* mRNA expression is prominent in hippocampal plate, diencephalon, cerebral cortex, and midbrain during embryogenesis [22].

Signaling molecules establish their effects on patterning and cellular differentiation through transcription factors, such as Foxa2 and Foxg1. Foxa2 is a member of Foxa family transcription

factor involved in development and metabolism. The midbrain dopaminergic neuron progenitor formation is triggered by Foxa2[24]. *Foxa2* expression is induced by Shh signaling pathway through GLI. Binding of Foxa2 to Shh enhancer attenuates Shh signaling through feedback loop [25]. Foxg1 is a winged-helix transcription factor. It is expressed in various nervous cell types and tissues. Studies showed that Foxg1 has a crucial role in the telencephalon specification, patterning, differentiation, and maintenance of mature neurons [26].

Our current pharmacological knowledge of natural products menacing role in pregnancy is limited. This research can be seen as an endeavor for unraveling the gene expression alteration in genes responsible for neurodevelopment in fetuses when saffron is misused during pregnancy. Concerning the failure to abort through folk practice is associated with an increased risk of fetal abnormalities, teratogenicity evaluation via molecular assessment is of importance.

2. Materials and Methods

2.1. Plant material

The *C.sativus* dried stigmas were purchased from Saharkhiz Company (Iran). The extract was prepared according to Premkumar *et al.* [27]. Briefly, the stigmas were finely ground to powder, and soaked in double distilled water and homogenized. After one hour, it was centrifuged for 10 min at 2000 rpm. The debris was removed and the supernatant was collected to use for the experiment. The weight of dried stigmas (mg) used to prepare 1 ml extract was the basis of dose calculation.

2.2. Test animals

NMRI mice (male: female ratio of 1:2) ten-week-old weighing 25-30 g were provided by Pasteur Institute of Iran. The experiment was carried out based on the guidelines of the Publication Principle of Laboratory Animal Care (NIH publication n. 86-23, revised 1985), by the ethical code obtained from Ethical Committee of Tehran Central Branch, Islamic Azad University, Tehran, Iran (IR. IAU.CTB. REC.1400.030 and 1400.018). The animals were allowed to acclimate under standard environmental conditions (12:12 h light/dark, 50±5% humidity, and 20-23 ˚C temperature), and access to food and water *ad libitum*. When the mice acclimated to the laboratory environment, 1 male: 2 female mice were placed in a cage. In the next morning, the copulation was detected by observing the vaginal plug. The day of plug observation is considered the first day of pregnancy. Subsequently, pregnant mice were divided in four groups (n=6): Group 1: normal control animals and three experimental groups: Group 2: 25 mg/kg saffron-treated animals, Group 3: 50 mg/kg saffron-treated animals, and Group 4: 100 mg/kg saffron-treated animals. Experimental groups were daily administered saffron aquatic extract by gavage during 7 to 12 days post coitum (dpc).

2.3. Sample collection

Mice were anesthetized and sacrificed by cervical dislocation (50 mg/kg) on 17 dpc. The fetuses were removed from the uterus. The macroscopic characteristics (number of fetuses, weight, crown-rump length, head length, tail length, and motor organs) were measured. To analyze the RNA expression, the head of fetuses were removed and saved in RNAlater (Sigma Chemicals Corporation, USA) and saved at -80 ˚C until molecular analysis.

2.4. Histology

After macroscopic analysis of fetuses, their skulls were opened by forceps and

the brain was completely removed. The brain extraction and preparation were done according to Rodgers *et al.* [28]. Horizontal sections were cut by microtome (DIAPATH, Italy). Finally, slides were stained with hematoxylin and eosin and histography was done by light microscopy at 40x magnification.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from brain samples by RiboEx Total RNA Kit (GENE ALL, South Korea) according to the manufacturer's protocol. Extracted RNA was qualitatively and quantitatively evaluated by 1.5% gel electrophoresis and spectrophotometry with NanoDrop (Berthold, Germany), respectively. Samples with a 260/280 ratio of 2 were used for the cDNA synthesis.

The reverse transcription was performed by cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran). Primers were designed by Oligo7 software, and their sensitivity was confirmed by NCBI BLAST (Table 1). The gene *Gapdh* was selected as the internal control in the current study. The cDNA was used as the template in real-time PCR (Rotor-Gene 6000, Corbett Research, Australia). The amplification condition was as follows: Initial denaturation at 95 ˚C for 15 minutes, 30 implication cycles included 15 seconds at 95 ˚C and 30 seconds at 60 ˚C (for *Fgf-8, FoxA2, FoxG1*, and *Gapdh*), and 30 seconds at 54 ˚C for *Wif1*, and final melting at 50-99 ˚C for 1 minute. $2^{-\Delta\Delta Ct}$ was used to calculate the fold change of gene expression in comparison to *Gapdh* gene as the internal control.

Table 1 Sequences of primers used for real-time PCR

Primer	Sequence (5'→ 3')	Tm (˚C)	Product size (bp)
Fgf8-F	CCGGACCTACCAGCTCTACA	60.4	220
Fgf8-R	GCCTTTGCCGTTGCTCTTG	60.37	
FoxA2-F	ATGCACTCGGCTTCCAGTAT	59.17	104
FoxA2-R	CTCACGGAAGAGTAGCCCT	58.11	
FoxG1-F	AGAACGGCAAGTACGAGAAGC	60.14	145
FoxG1-R	TGCTTGTTCTCGCGGTAGTAG	60.14	
Wif1-F	CCCAACAAAGAATGCCAG	53091	194
Wif1-R	ACCAACTTGAACAACTGATG	54.03	
Gapdh-F	GAAGCTTGTCATCAACGGGA	58.19	180
Gapdh-R	GAAGGGGCGGAGATGATGAC	60.25	

Statistical analysis

All the experiments were done in triplicate and the results were reported as mean ± standard deviation (SD). One-way ANOVA was used to calculate the differences followed by Tukey post hoc test. GraphPad Prism (version 8) was used to calculate statistical analysis. $P<0.05$ was considered statistically significant.

3. Results

Macroscopic observation

Fetal viability was comparable between the saffron extract- treated groups and the control group. All dams in the control group harbored 13.38±3.07 fetuses. Dams treated with 100 mg/kg saffron extract showed cystic, ovarian hyperemia, and their fetuses were undeveloped and absorption sites existed. The treatment with 50 mg/kg saffron extract led to the maintained

pregnancy only in one of the dams with 6 fetuses.

At 25 mg/kg dose, all dams averagely carried two fetuses. The fetuses' weight, crown-rump length (CRL), head, fore limb, hind limb, and tail length in control and treated groups are represented in Table 2. Although the change was observed in most measured characteristics of fetuses exposed to 50 mg/kg saffron extract, the significant difference was in CRL (P<0.01). Due to the treatment of 25 mg/kg saffron extract, the average fetal weight increased (P<0.001), while the CRL and hand length decreased (P<0.05).

The length of the tail decreased in 25 mg/kg treated group in comparison to both control and 50 mg/kg saffron-treated groups (P<0.001).

Table 2 Macroscopic parameters of fetuses in control and treated groups[a]

	Control	25 mg/kg	50 mg/kg	100 mg/kg
Number of fetuses	81	12***	6***	0
Mean weight ± SD (g)	0.92 ± 0.12	1.06 ± 0.02***	0.92 ± 0.09^	-
Mean CRL[b] ± SD (mm)	21.59 ± 1.44	20.37 ± 0.260*	19.56 ± 0.82**	-
Mean head length ± SD (mm)	7.32 ± 0.56	6.99 ± 0.44	7.55 ± 0.32	-
Mean tail length ± SD (mm)	11.53 ± 1.08	9.95 ± 0.17***	11.94 ± 1.02^^^	-
Mean hand length ± SD (mm)	8.17 ± 1.47	6.96 ± 0.2*	7.93 ± 0.86	-
Mean foot length ± SD (mm)	9.08 ± 1.67	8.22 ± 0.11	8.92 ± 1.1	-

[a] Pregnant mice in test groups were treated with 25 mg/kg, 50 mg/kg, and 100 mg/kg doses of saffron aquatic extract and controls received normal saline through gavage administration during 7-12 dpc. They were euthanized at 17 cdpc.
[b] CRL: Crown-rump length
[c] dpc: days post coitum
** P< 0.05 compared to the control group.*
*** P<0.01 compared to the control group.*
**** P<0.001 compared to the control group.*
^ P<0.05 compare to the 25 mg/kg treated group.
^^^ P<0.001 compare to 25 mg/kg treated group.

Histological analysis

Sections of the forebrain, midbrain, hindbrain, and cerebellum were investigated. The findings presented similar histological characteristics in treated groups compared to control animals. The brain tissue in all three groups was well-formed. The cortex in the two treated groups was well-stratified as the control group (Figure 1).

Well-differentiated layers, including the subventricular and ventricular zone, intermediate zone, subcortical and cortical plate, and marginal zone were observed. Both halves of the thalamus were merged in the midline and formed the intrathalamic junction.

The pituitary gland was well-differentiated. The pineal gland was obviously developed.

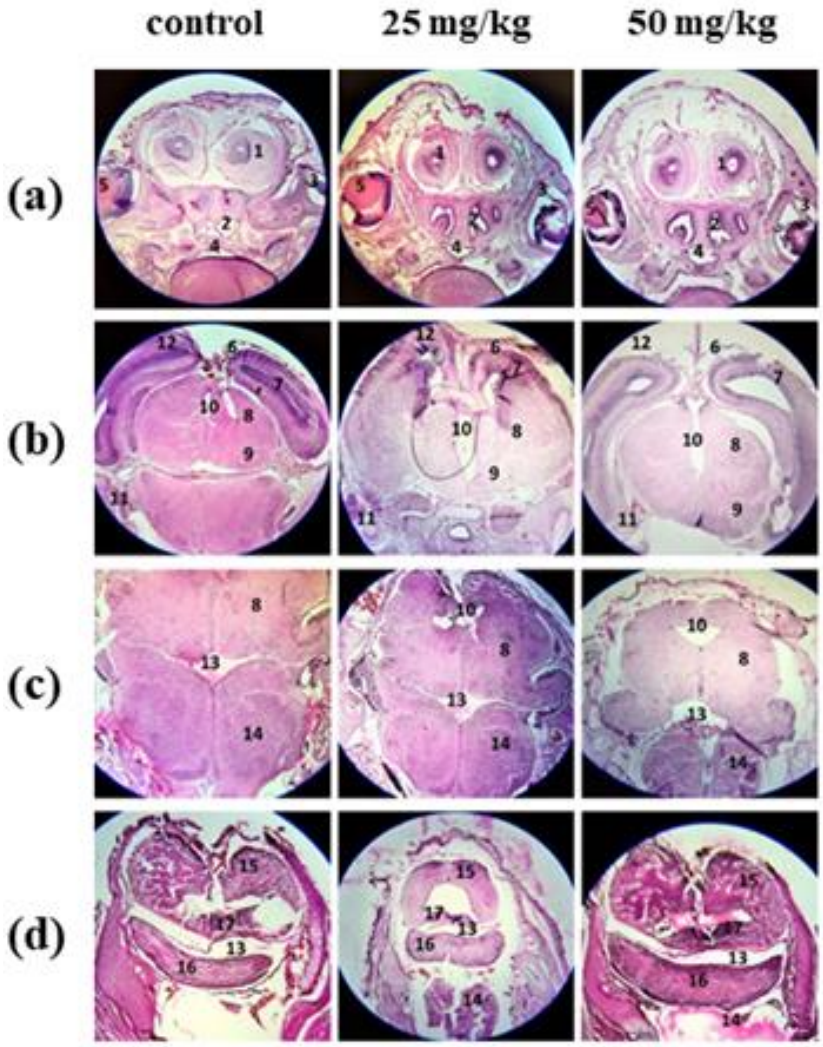

Figure 1. H & E images of (a) the forebrain, (b) the midbrain, (c) the hindbrain, and (d) the cerebellum in control, 25 mg/kg saffron-treated, and 50 mg/kg saffron extract- treated groups. (1. Olfactory lobe, 2. Nasal cavity, 3. Optic vesicle, 4. Nasopharyngeal canal, 5. Lens, 6. Telencephalon, 7. Hippocampus, 8. Thalamus, 9. Hypothalamus, 10. Third ventricle, 11. Olfactory lobe, 12. Lateral ventricle, 13. Fourth ventricle, 14. Quadruple ridges, 15. Medulla, 16. Cerebral pons, and 17. Cerebellum)

Gene expression analysis

We investigate *Foxa2, Foxg1, Fgf8,* and *Wif1* mRNA expression (Figure 2). 25 mg/kg treatment of saffron extract upregulated the expression of *FoxA2, Foxg1,* and *wif1* (P<0.0001) in the brain tissue of fetuses. 50 mg/kg intervention led to significant raise of *Foxa2* and *Wif1* in comparison to the control animals (P<0.0001) and 25 mg/kg administration (P<0.01 and P<0.001, respectively). In the case of *Foxg1* expression, 50 mg/kg did not significantly change *Foxg1* mRNA level compared to the control group, hence the animals treated with 50 mg/kg extract showed less *Foxg1* mRNA level in comparison to the other treated group (P<0.0001) (Figure 2a, b, and c).

The expression of *Fgf8* mRNA in 50 and 25 mg/kg treated groups was down-regulated than control group (P<0.0001 and P<0.001, respectively).

A significant difference was observed between treated groups (P<0.0001) (Figure 2d).

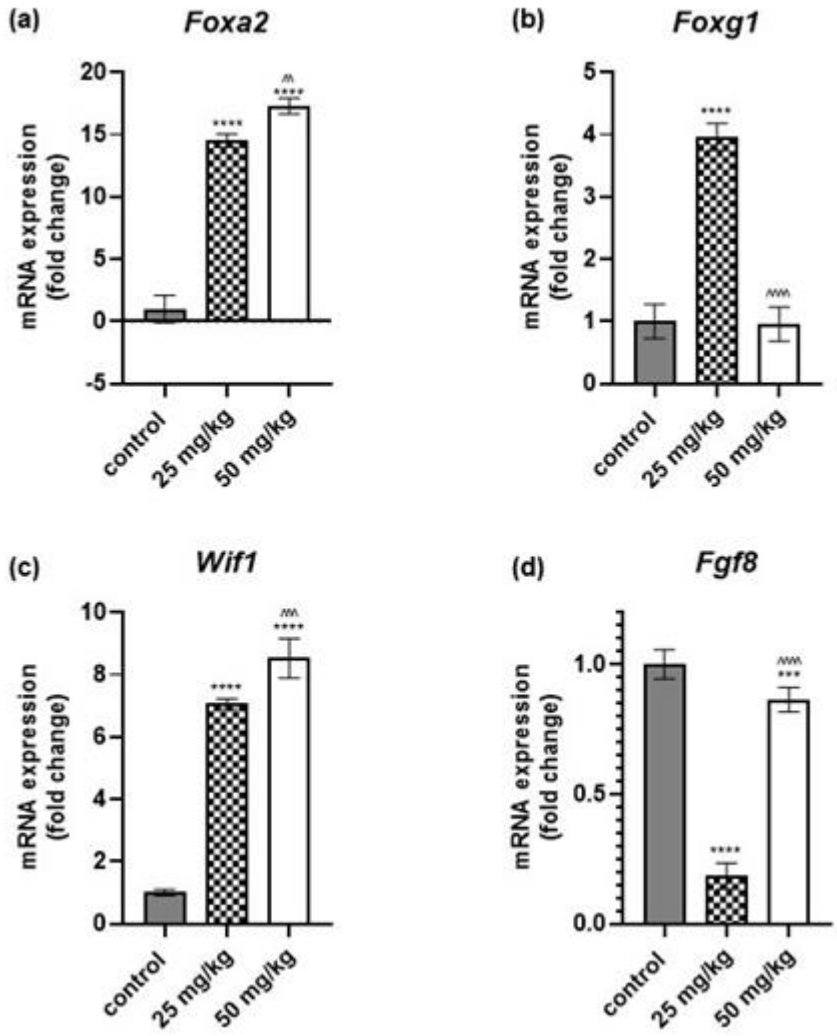

Figure 2 Effects of 25mg/kg and 50 mg/kg of the aquatic saffron extract on (a) *FoxA2* mRNA level, (b) *FoxG1* mRNA level, (c) mRNA level of *Fgf8*, and (d) mRNA level of *Wif1*

**** P<0.001 compared to the control group.*
***** P<0.0001 compared to the control group.*
^^^ P<0.001 compared to the 25 mg/kg-treated group.
^^^^ P<0.0001 compared to the 25 mg/kg -treated group.

4. Discussion

The stigma of *Crocus sativus* has been considered a phytomedicine and spice since ancient times. In recent years, saffron is widely used based on scientific studies investigating its protectiveness against aging and cancer [8,9,11]. Among all studies on saffron and its constituents, only limited studies investigated its adverse potential on pregnancy and its capability of teratogenicity [12,14].

Although these studies reported the anatomical and morphological consequences, they did not consider molecular actors causing saffron adverse compatibility on the fetus. Hence, in the current study, we tried to investigate expression alterations of four genes playing crucial roles in brain development. Accordingly, we used three doses of saffron aquatic extract (25, 50, and 100 mg/kg).

The results of the present study indicated that saffron extract reduced the number of fetuses compared to control animals in a dose-dependent manner. 100 mg/kg treatment resulted in the absence of fetuses in the uterine. 25 mg/kg treatment raised the fetal weight in comparison to the normal group and 50 mg/kg treated group. On the other hand, CRL dose-dependently alleviates in both treated groups in comparison to controls. Other significant morphological differences were observed in the length of tail and hand, in which the difference was more in 25 mg/kg treated group, while the results of 50 mg/kg treatment were approximately equal to the results of control animals. The histopathological results showed no significant difference in both examined groups in comparison to the control group and all three groups, brain tissue was well formed and stratified.

In some studies, aimed to evaluate herbal teratogenicity, the fetal weight alleviated [29]. The raise of fetal weight in treated groups was not in accordance with Moallem *et al.* [12]. The dissimilarity can be due to the intervention method (gavage vs. IP). In addition, they used crocin and safranal as the main phytochemicals of saffron. If this subject considers, it shows a dose-dependent effect of saffron on fetal weight. In another study led by Dashti *et al.*, the increase in fetal weight of intervention groups was observed. They deduced that this could be due to the different weighing times [30]. The decrease in tail size observed in 25 mg/kg treated group is supported by Al-Qodsi study [31].

This phenomenon may be caused by the disruption of fetal development induced via aquatic saffron extract. Brain formation is a process of outstanding importance to achieve an organ with a specialized combination of activities in a complex texture. Histological results of the current study present no difference in brain formation among treated groups and controls. We also assess the expression of genes important in fetal brain development. Various signals are merged to provide a correct spatio-temporal organization of the brain including Shh, Wnt, and Fgf. Shh regulates the expression of *Foxg1* and *Foxa2* directly and through Otx2, respectively. The expression of *Foxg1* increased 3-fold change in 25 mg/kg treatment, while its expression in 50 mg/kg treated group was equal to normal controls. Studies demonstrated that *Foxg1* expression alteration affects the function and formation of the cerebral cortex. The normal cortical thickness observed in this experiment could be the result of the increase in the expression of *Foxg1*. In addition, Foxg1 controls inhibitory and excitatory inputs in the cortical circuits. Augmented expression of *Foxg1* is a feature in glioblastoma and triggers the dedifferentiation of neurons to neural stem cells. *Foxg1* suppression reduces the proliferation of GABAergic progenitor cells.

Hence, Foxg1 is a mediator in the development of inhibitory neurons. It can be concluded that Foxg1 dysregulation is a primary mediator of Autism spectrum disease [26]. The *Foxa2* expression was ascending in a dose-dependent manner. *Foxa2* expression exhibits a profound coordination between the placenta and fetal brain elucidating a functional relation between placenta and fetal brain (i.e. brain- placental axis).

Dhakal *et al.* showed that the lack of *Foxa2* expression in the uterus globally dysregulated gene expression in both brain and placenta based on the fetal gender [32]. In our study, *Foxa2* expression included a high standard deviation number in controls, which can be explained by the sex-biased *Foxa2* mRNA level difference**.** Studies

introduced saffron as a promising herbal medication against Parkinson's disease (PD) because of its antioxidant and anti-inflammatory properties [33]. In fetuses, Foxa2 pivotally targets key genes in the development of midbrain dopaminergic (mDA) neurons [24]. *Foxa2* is also expressed in adult mDA neurons to promote survival and protect against toxic insults. During aging and neuronal degeneration, Foxa2 level alleviates [34]. It can be possible that the positive effect of saffron on PD is because of *Foxa2* upregulation. Analyzing *Foxa2* expression in PD mouse models treated with saffron can be considered for further studies. The other gene which is regulated by Shh signaling pathway is *Wif1* which is an antagonist of Wnt. Its expression increased dose-dependently, which could inhibit Wnt canonical pathway. Some studies revealed that crocin (the major phytochemical of saffron) decreases the activation of Wnt signaling pathway in cancerous cells [35]. That could occur through *Wif1* expression increase. However, there is a need for more investigations on saffron and its components against canonical and non-canonical- Wnt pathway. It has been shown that the developing brain regions are differentially sensitive to Fgf8 signaling [36]. In the current study, the *Fgf8* expression acted differently in the two concentrations of treatment. Its expression decreased at 25 mg/kg, while at 50 mg/kg increased. Foxg1 directly upregulates *Fgf8* expression while Shh does it indirectly through Gli3. Both Foxg1 and Fgf8 are needed to develop telencephalon. Our result indicated somehow opposite expression pattern of *Foxg1* and *Fgf8* which may be deduced that in this experiment Foxg1 did not directly regulate *Fgf8* expression and another pathway (e.g., Gli3 upregulation) caused the reduction. In this study, we tried to investigate the expression changes in four genes. Although it is clear that the saffron affected not only their expression, it also influenced other genes. It is better to use high throughput techniques to assess the saffron effect on neurodevelopment in the fetus.

Conclusion

In conclusion, the administration of 100 mg/kg aquatic saffron extract led to the loss of all fetuses, 50 and 25 mg/kg treatments reduced the number of fetuses drastically. Fetal brain histological investigation showed similar tissue formation in treated groups in comparison to control animals. Gene expression evaluation of *Foxa2* and *Wif1* exhibited a dose-dependent increase. *Foxg1* augmentation was significant only in 25 mg/kg treatment. *Fgf8* mRNA level decreased in response to both treatments, but more significantly in 25 mg/kg treatment. Our results might be a benchmark for more studies on saffron effects on fetal brain development. Further molecular investigations should be conducted about saffron extract administration during the whole gestation period. According to the current findings, it is advisable to examine the clinical applicability of *C. sativus* and its phytochemicals on neurodegenerative diseases.

Consent for publications

All authors agree to have read the manuscript and authorize the publication of the final version of the manuscript.

Availability of data and material

Data are available on request from the authors.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Ethics approval and consent to participate

The ethical approval was certified by Islamic Azad University Tehran Central Branch (Ethic Committee Reference No. IR. IAU.CTB. REC.1400.030 and 1400.018).

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