



# Pomegranate seed extract reduces ischemia induced anxiety in male rats

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## ABSTRACT

**Introduction:** Ischemia-associated depression and anxiety may occur due to brain damage caused by oxidative stress. A number of reports indicated that treatment with herbal plant extracts with antioxidant properties could lead to a significant reduction in Ischemic complications. The aim of this study was to evaluate the behaviors of ischemic rats through animal models of anxiety and exploratory behavior (open field) to determine the efficiency of pomegranate seed extract (PGSE) as antioxidant.

**Methods:** In this study, 21 male Wistar rats (250 ± 20 g) were randomly divided into 3 groups with 7 in each: 1) Control; 2) Ischemic; 3) Ischemic plus PGSE (14 days). In order to create ischemia/hypoperfusion, carotid arteries were ligatured and cut bilaterally. Then, plus maze and open-field tests were used for the measurement.

**Results:** Ischemic rats showed a significant increase in anxiety or decrease approach and reduction locomotor activity compared to control group. Fourteen days administration of PGSE significantly improved the immobilization and ischemia-induced anxiety.

**Conclusion:** PGSE exhibits therapeutic potential for anxiety and depression, which is most likely related, at least in part, to its antioxidant and free radical scavenging actions.

### Implication for health policy/practice/research/medical education:

Anxiety is a disorder caused by ischemia. Pomegranate seed extract (PGSE) can reduce anxiety by reducing oxidative stress induced by ischemia. Hence, it might be used to reduce this problem.

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## Introduction

The brain has high demand for glucose oxidation in order to synthesize adenosine triphosphate (ATP). When oxygen supply is disrupted, pathological changes occur (1,2). Decreased blood flow to the brain (cerebral hypoperfusion) reduces oxygen supply to the brain as a result of cerebral ischemia (3). Mammalian brain is very sensitive to oxygen deficiency and even a short time of hypoxia can cause irreparable injury or even death. Although oxygen is vital, the production of oxygen-induced reactive species through respiration and metabolism in the brain causes toxin generation in the brain, which is due to the natural oxidative vulnerability of the brain (4). In neurodegenerative diseases, the continuation of cerebral hypo-perfusion is associated with the severity of cognitive impairment (5,6). Anxiety disorders are the most prevalent mental health conditions. Anxiety is a protective feeling, but too much anxiety is very debilitating and can

demonstrate itself anxiety disorders (7). Although there are effective treatments for anxiety-related disorders, most patients suffering from anxiety do not react to classical anti-anxiety drugs and also demonstrate the side-effects, which could limit their compliance with the prescribed regimen. In recent studies, a close relationship has been observed between oxidative stress and anxiety both in humans who suffer from anxiety disorders (obsessive compulsive disorder and panic disorder) and in animals and humans that have high specific anxiety (8,9). Currently, large attention has been paid to the use of natural antioxidants for body protection, especially the brain tissue, which protects it from oxidative damage caused by free radicals (10). The extracts derived from different parts of pomegranates are rich in phenolic compounds. Also, skin extract and seed oil of this fruit have powerful antioxidant activities that could be used to deactivate free radicals (11). Today, in addition to being

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a fruit, the medicinal properties of various pomegranates have been widely considered by researchers from different countries (12). According to the above-mentioned cases, we decided to investigate the effect of pomegranate seeds extract on anxiety and motor activity in an ischemia model.

## Materials and Methods

### Animals

All the experiments of this study were performed using male Wistar rats obtained from Breeding Centre and Animal House of Ahvaz Jundishapur University of Medical Sciences within the weight range of 200-250 g. The animals were maintained in the following standard conditions:

20 ± 2°C, 12-hour photoperiodic cycles (light starting from 7 AM), and adequate access to intensive livestock food produced by Pars Co. in Tehran and Chavdanh Co. in Shahreza, Isfahan, and treated freshwater of Izeh city. The animals were kept in 4 standard cages in groups of 4 in Breeding Centre and Animal House of Ize Islamic Azad University. To perform the test, the animals were divided into the following groups:

1. Healthy group without ischemia induction and receiving drugs (control)
2. Ischemic hypoperfusion group without receiving drugs (ischemia)
3. Ischemic hypoperfusion group treated with total extract of pomegranate seed (400 mg/kg) for 14 days by intragastric administration or gavage.

### Brain hypoperfusion ischemia model

Animals were anesthetized using ketamine/xylazine (50/5 mg/kg, i.p). A neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vagus nerve. Carotids were occluded with three days interval between interventions, the right common carotid being the first to be assessed and the left one being occluded three days later (13). Afterwards, the animals of each group were treated by gavage for 2 weeks with pomegranate seed extract (PGSE).

### Pomegranate seed extract preparation

Pomegranate fruits (the products of Shivand gardens) (*Punica granatum L.*) which were authenticated by a botanical expert from Izeh Islamic Azad University were deseeded and their juice was extracted. Then, the seeds were dried and milled and 72% alcohol was added to the milled seeds. The obtained mixture was stored in a beaker for 3 days and mixed in the mornings and evenings. They were spread on a flat surface after 3 days to dry. Finally, the obtained extract was collected (purity percentage of the extract was 17%), dissolved in distilled water, and injected into the rats by gavage for 14 days (14,15).

### Behavioral tests

#### Open-field test

In this study, we used a white box with the size of 72\*72

cm and height of 36 cm. The field device included 16 small parts with black lines and one central part with red lines. In this method, animal passing from each part to another by all of its four motion members was considered a single movement. The number of movements in the margin, number of movements to the center, and frequency of mice excretion were recorded by a camera from the superior surface of the device and then evaluated. The sum of the test times, kept in each chamber during a period of 5 minutes, was calculated for each mouse. Then, the mouse was removed from the field, cleaned and dried, using a piece of cotton dipped in alcohol (16).

#### Plus maze

To assess anxiety level, a device called elevated plus maze was used that is a standard model to assess the anxiety level of rodents. It was made of wood and had one open arm (50 × 10 cm each with a 5 mm edge), 2 closed arms (40 × 50 × 10 cm each), and a central pan (10 × 10 cm). Its height was 70 cm from the ground. The animal was put on the central area facing an open arm in the device. Within 5 minutes during which the animal was freely moving in the device, the number of entry into the open and closed arms and the total time spent in open and closed arms were measured. Entry into the open or closed arms occurs when all four feet of the animal were placed on the considered arm (17). Using the collected data, the percentage of open arms entries to total arms entries (%OAE), the percentage of time spent on the open arm to total spending time on the all arms (%OAT).

### Statistical analysis

Data of this study were presented as mean ± SEM and were analyzed in Microsoft Excel and SPSS using one-way analysis of variance (ANOVA) and LSD test. Difference in the results between different groups with at least  $P < 0.05$  was considered statistically significant.

## Results

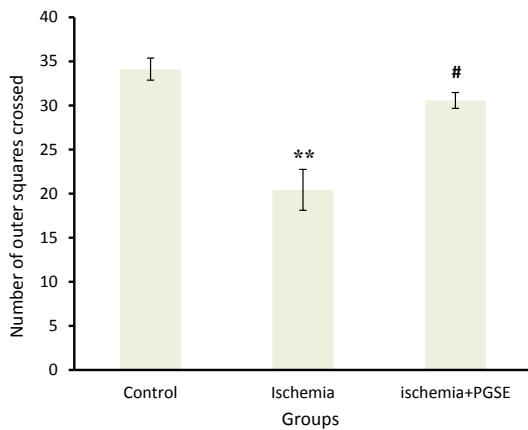
### Open-field

The findings showed a significant increase in terms of anxiety level and decrease in locomotor activity among ischemic mice compared with the control group at  $p < 0.01$  and  $P < 0.001$ , respectively. Also, 14 days administration of PGSE to the ischemic mice led to reduced level of anxiety and exploratory behavior and increased locomotor activity compared with the group without treatment at  $P < 0.05$  and  $P < 0.01$  (Figure 1).

### Maze test

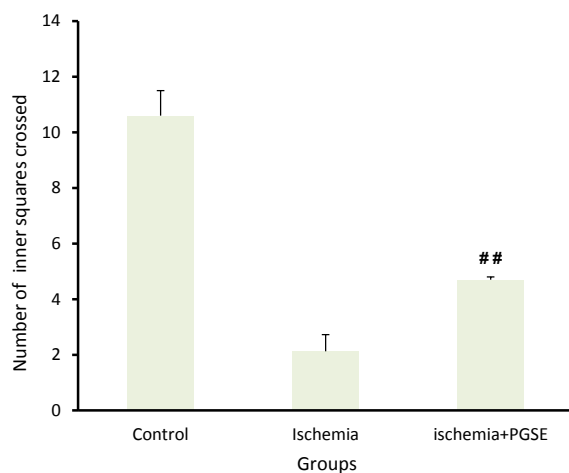
Given the frequency of stool excretion, often used as a measure of anxiety, in this study, the frequency of stool excretion in the ischemic group was increased ( $P < 0.001$ ) compared with the control group, while a significant reduction was observed in the frequency of stool excretion in the ischemic group receiving PGSE (Figure 2).

Significant increase in two parameters of A (percentage of time spent in the open arm) and B (percentage of entry



**Figure 1.** Mean  $\pm$  SD of the number of passing time through external squares between the control, ischemic, and ischemic groups orally receiving 400 mg/kg of pomegranate seed extract (PGSE) for 14 days.

\* Significance compared with the control group. #Significance compared with the ischemic group (one-way ANOVA post hoc LSD test, # $P < 0.05$ , \*\* $P < 0.01$ ).



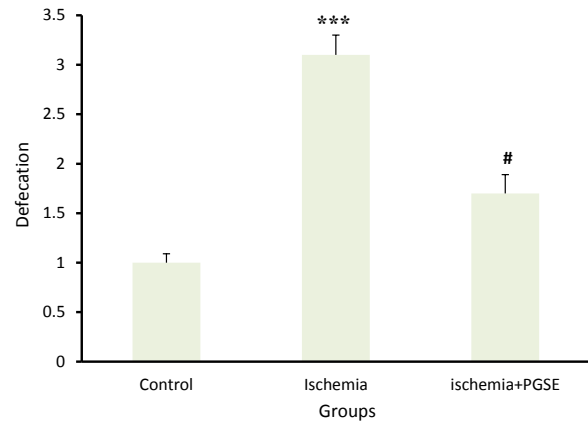
**Figure 2.** Mean  $\pm$  SD of the number of passing through internal squares among the control, ischemic, and ischemic groups receiving 400 mg/kg of pomegranate seed extract (PGSE) orally for 14 days.

\* Significance compared with the control group. #Significance compared with the ischemic group (one-way ANOVA post hoc LSD test, ## $P < 0.01$ , \*\*\* $P < 0.001$ ).

into the open arm) showed the reduced level of anxiety in this test. Ischemia reduced the amount of time spent in the open arms ( $P < 0.001$ ) and the number of entry into this arm ( $P < 0.01$ ), which reflected anxiety in studied group compared with the control group. The results also showed that administration of PGSE increased the percentage of time spent on the open arm and percentage of entry into this arm at  $P < 0.05$ , which indicated anxiety reduction in the group receiving PGSE compared with the ischemic group (Figures 3 and 4).

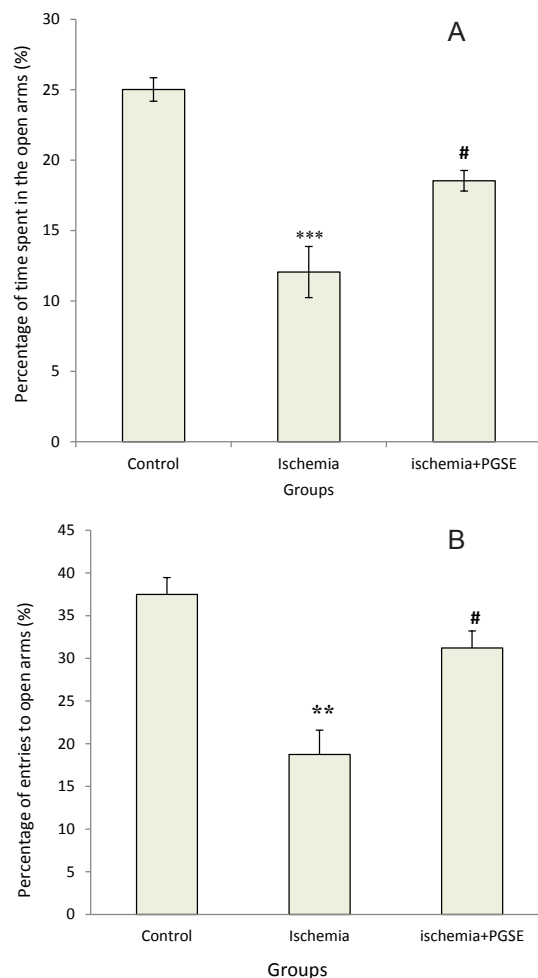
## Discussion

Generally, factor analysis using open-field tests is the result of 3 factors described in 1997 by Ramusu et al—an anxiety



**Figure 3.** Mean  $\pm$  SD of the frequency of excretion between the control, ischemic, and ischemic groups orally receiving 400 mg/kg of pomegranate seed extract (PGSE) for 14 days

\* Significance compared with the control group. #Significance compared with the ischemic group (one-way ANOVA post hoc LSD test, # $P < 0.05$ , \*\*\* $P < 0.001$ ).



**Figure 4.** Mean  $\pm$  SD of A (percentage of time spent on the open arm), B (percentage of entry into the open arm) between the control, ischemic, and ischemic groups orally receiving 400 mg/kg of pomegranate seed extract (PGSE) for 14 days.

\* Significance compared with the control group. #Significance compared with the ischemic group (one-way ANOVA post hoc LSD test, # $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

behavior or avoidance/approach (moving in the central square), scores of locomotor activity (moving in marginal squares), and stool excretion. Results of this study showed that ischemia was able to decrease locomotor activity and increase anxiety, while PGSE could cause anxiety reduction and increase motor activity in the animal models of ischemia. Brain vulnerability to oxidative damage corresponds to the theory that anxiety can occur directly through oxidative stress. When oxidative stress causes anxiety, antioxidants may have therapeutic potential in the meantime. In addition, a combination of antioxidants and anxiety reduction drugs can be a useful treatment for the patients with anxiety, because anxiety disorders are associated with oxidative stress. It has been known that vitamin C has an antidepressant effect along with conventional antidepressant drugs that are administered in effective doses (18,19). While the production of reactive oxygen species (ROS) dominates the defense system of the brain, the lipid-rich brain structure can be susceptible to lipid peroxidation that creates a chain reaction of free radicals, which can reduce membrane fluidity and damage membrane proteins leading to the loss of receptors, enzymes, and ion channels and eliminating the membrane integration that ultimately causes cell death. In addition to oxidative damage to proteins, lipids, and nerve cell membranes (neurons), oxidation can also occur in other sensitive sectors and transmitter of biological nucleic acids. As a result, oxidative stress can change neural transmission, neuronal function, and overall brain activity (20). The comparable brain circuits involved in anxiety and chemically similar neural substrates (like GABA, serotonin), among other factors, cause rodents to become a good model for the study of anxiety in humans (21). High level of anxiety is associated with remarkable production of ROS in the peripheral blood lymphocytes, granulocytes, and monocytes in mice compared with low levels of anxiety. These results confirm the relationship between level of intracellular ROS blood cells and anxiety-related behavior in mice (8). In addition, previous studies have observed a significant relationship between specific anxiety and formation of ROS in the monocytes of participants (21,22). Berry et al showed that mice demonstrated anxiety behaviors with the increasing age, which could be probably due to the accumulation of oxidative damage, as one of the characteristics of aging process in animals. In addition, they showed that drainage of longevity gene *p66Shc* that decreased oxidative stress and increased longevity could reduce anxiety-related behavior (23). Polyphenols have shown their ability to relieve anxiety-related behavior in rodents (23). Some polyphenols have medicinal conditions that show a minor conflicting activity, which may show effects such as anxiety reduction without side-effects. Antioxidants obtained from a normal diet can prevent the spread of anxiety (24). Pitozzi et al found that the older rats, fed on foods containing olive oil, which is naturally rich in antioxidants, for one year demonstrated less anxiety than the aged mice that were not fed on the foods containing olive oil or corn

oil (25). Pomegranate juice is rich in phenolic compounds, which is higher than many other fruit juices. Phenolic compounds form an important group of plant compounds as secondary metabolites that are produced in response to the environmental stress. Due to having hydroxyl groups, these compounds could neutralize free radicals and act as electron or hydrogen donors (26).

### Conclusion

Oral administration of hydro-alcoholic extract of pomegranate seeds, once a day for 14 days, significantly improved the disorder caused by cerebral ischemia on stress and anxiety behaviors. Since PGSE contains phenolic compounds including ellagic acid in free and bond forms as well as other flavonoids, subsequent disorders of ischemia disorders can be improved using the method of sweeping oxidants and free radicals produced by the brain's ischemia.

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### Authors' contributions

SG: Design of the study and laboratory methods, preparation of PGSE and laboratory methods. SV: Preparation of the paper draft, statistical analysis and help to laboratory methods. Both authors read the final version of the article and confirmed its publication.

### Conflict of interests

The authors declared no competing interests.

### Ethical considerations

The experiments were confirmed by Ethical Committee in Vice Chancellor of Research of Islamic Azad University. Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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