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Effect of *Ceratonia siliqua* extract on the density of hippocampus dark neurons in adult male mice

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Abstract
Studies show nutrition effects on function and histology of the brain. Based on our search, there is no study on the effects of *Ceratonia siliqua* on dark neurons in the brain. Therefore, this study investigated the effects of different doses of carob extract on the dark neurons in hippocampus of adult male Balb/c mice. In this experimental study, 32 adult male Balb/c mice were randomly divided into five groups (sham and carob 1 to 3). Carob 1 to 3 groups received intraperitoneally doses of 200 mg/kg, 400 mg/kg and 800 mg/kg of carob extract for 14 days. Then, the brains were stained with toluidine blue and the mean density of the dark neurons was counted in the hippocampus. SPSS software and one-way ANOVA was used to analyze the data. The mean number of dark hippocampal neurons was 64.71 ± 1.68 N/mm^2 in sham group and 68.71 ± 0.9 N/mm^2 in carob 1 group. Statistical analysis showed the administration of 200-800 mg/kg of carob extract does not change the number of dark neurons in the hippocampus of the adult mice compared to the sham group.

Keywords: carob, mouse, hippocampus, dark neuron

1. Introduction
Ceratonia siliqua is an herb from the fabaceae family. Fabaceae is an important part of the vegetation of tropical, subtropical and temperate regions around the world (Batlle & Tous, 2009; Seczyk *et al*. 2016) [5, 19]. The carob tree is an evergreen tree with a height of 7-12 meters, which it does not produce any fruit in the first 15 years of its life (Batlle & Tous, 2009; Seczyk *et al*. 2016) [5, 19]. The carob is one of the most useful trees in Mediterranean region that grows in Iran in areas like Fars province. The fruit is brown in color, long and curved, with about 12 to 16 seeds very hard like lentils. It contains 40% to 50% carbohydrates, and about 5% fat and protein (Batlle & Tous, 2009; Seczyk *et al*. 2016) [5, 19].

Studies show that the carob extract protects the harmful effects of alcohol in the gastrointestinal tract (Rtibi *et al*. 2015) [17]. In addition, the carob has antioxidant (Custódio *et al*. 2011) [9], anti-bacterial (Meziani *et al*. 2015) [15], anti-cancer (Corsi *et al*. 2002) [6, 7] and anti-diabetic properties (Rtibi *et al*. 2017) [18]. Studies show nutrition effects on the structure and function of the brain. For example, the Mediterranean diet reduces the risk of Alzheimer's and Parkinson's disease by 13% (Rtibi *et al*. 2008). Also, there is a correlation between the deficiency of omega-3 fatty acids with an increase in Alzheimer's and Parkinson's (Parrott & Greenwood, 2007) [16]. The presence of trace elements such as zinc and copper in the diet is critical for the development of neurotransmitters, energy metabolism, and antioxidant defense of the brain (Jones *et al*. 2008) [14]. The administration of carob extract decreased depression in animal model (Agrawal *et al*. 2011) [1]. In addition, it influenced on peripheral benzodiazepine receptors (Avallone *et al*. 2007).

On the other hand, the presence of dark neurons is one of the most significant neurological damage in many diseases of the nervous system, such as epilepsy and ischemia. These severe basophils neurons are picnotic and small. The Dark neurons have compact, degenerate, dense and irregular chromatin (Kherani *et al*. 2008; Baracskay *et al*. 2008; Kiernan *et al*. 1998) [11, 12, 4]. Based on our search, there was not study to date on the effects of carob administration on dark neurons in the brain. Therefore, this study was conducted to determine the effects of different dosages of carob extract on the density of dark hippocampal neurons in adult Balb/c male mice.

2. Methods and Materials
This experimental study was performed on 32 adult male mice. Mice were under the standard condition in terms of temperature and light. Animals had free access to water and food. The mice randomly divided into 4 groups (sham and carob 1 to 3). The sham group received normal saline. Carob groups received intraperitoneally 200, 400 and 800 mg / kg of aqueous extract of carob for 14 days.

Carob Extract
100 g of carob powder was added to 1000 ml distilled water and it placed on a stirrer (Ika, Germany) overnight .Then passed through whatman filter paper (England) and placed at 40 ° C in water bath .Finally, the extract was dissolved in normal saline and injected intraperitoneally into mice (Vafaei *et al*. 2018) [21].

Dark neuron count
After deep anesthesia with chloroform (Merck, Germany), the brain was removed and placed in formalin solution (Merck, Germany). After the tissue processing, brain sections in thickness of 5 microns were prepared. The distance from each section to the next was 5 µm [17]. Toluidian blue (Merck, Germany) was used for staining dark neurons. For this purpose, 1 g of Toluiden blue powder was dissolved in 100 cc alcohol (Pars Teb, Iran) and stored in the refrigerator overnight. Then 25 ml of solution was dissolved with 250 ml of salt and distilled water and passed

through filter paper. After staining the hippocampal sections with Toluiden blue, slides were photographed with a digital camera and 40× magnification. Then, images were transferred to the computer. The mean numerical density of dark neurons per unit area of tissue was counted using the frame with the following formula (Bagheri-Abassi *et al*. 2015) [3].

$$N_A = \frac{\Sigma \bar{Q}}{\alpha/f.\Sigma p}$$

N_A: the number of cells per unit area, QΣ: the total number of counted cells, a/f: the surface area of each frame counting and ΣP: the total number of counted frames (Bagheri-Abassi *et al*. 2015) [3]

Statistical analysis

Data were expressed as mean ± standard deviation and entered into SPSS software **.**One-way ANOVA and tukey post hoc test were used to compare groups. P<0.05 was considered as a significant level**.**

3. Results

Investigation the hippocampus stained sections with Toluiden blue

Figure 1 shows the coronal sections of the hippocampus in the four groups using Toluidian blue staining. As shown, the mean numerical density of the dark hippocampal neurons in the control group was 64.71 ± 1.68 N/mm^2 in sham group and 68.71 ± 0.9 N/mm^2 in carob 1 group. The mean number of dark neurons in the hippocampus in mice received 400 mg/kg carob was 65.10 ± 3.01 cells per mm^2 and the mean number density of the dark neurons in the hippocampus of the mice received 800 mg/kg was 64.83 ± 2.42 cells per mm^2 (diagram 1-3). The statistical analysis showed no significant difference between carob1 (p=0.54), carob 2 (p=0.97), and carob 3 (p=1.000) groups compared to the sham group (p>0.05).

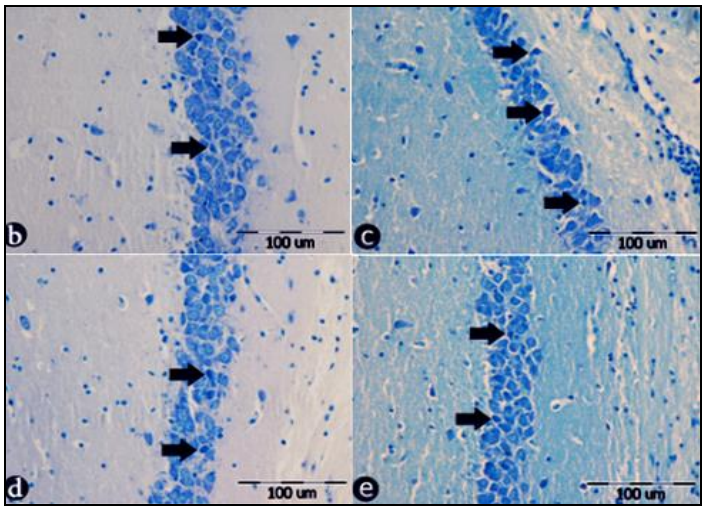

Fig 1: Photomicrograph of adult hippocampus in different groups that were stained with Toluidian blue **.**Dark neurons are marked with arrows. Magnification is 400 ×. b: sham group, c: carob group 200 mg / kg, d: carob group 400 mg / kg and e: carob group 800 mg / kg

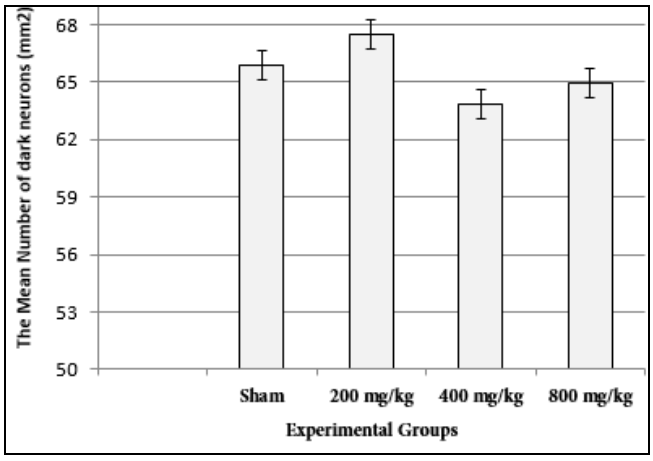

Fig 2: The mean number of hippocampal dark neurons in CA1 part of hippocampus in different groups

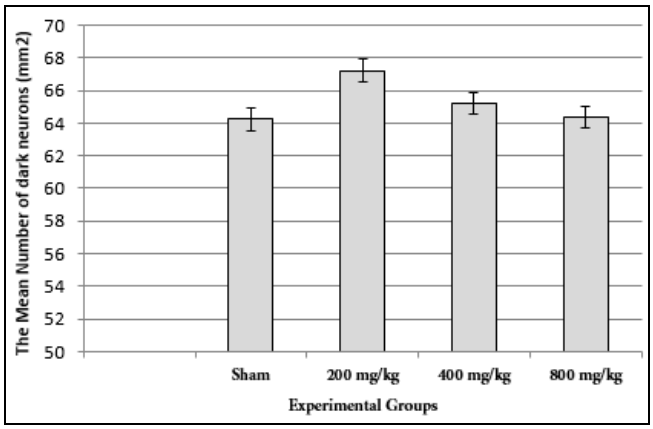

Fig 3: The mean number of hippocampal dark neurons in CA2 part of hippocampus in different groups

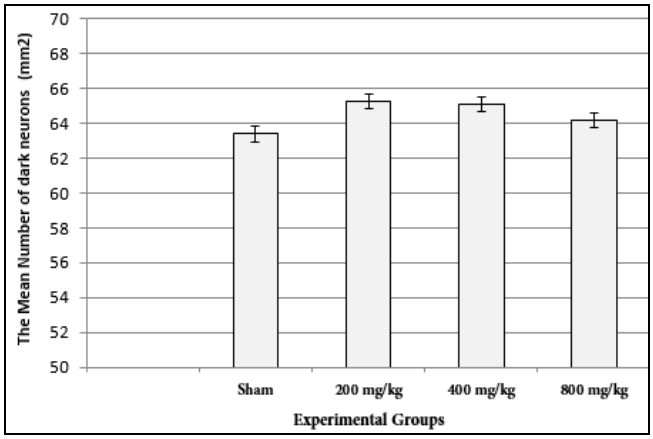

Fig 4: The mean number of hippocampal dark neurons in CA3 part of hippocampus in different groups

4. Discussion

The results of this study showed that there was not a remarkable difference between carob groups compared to the sham group. This may be due to the fact that injection-induced stress in the sham group and the carob groups and it caused an increase in the number of dark neurons in the hippocampus**.**

Studies show nutrition affects the structure and function of the brain. For example, the Mediterranean diet has reduces the risk of Alzheimer's and Parkinson's disease by 13% (Rtibi *et al*. 2008). There is a correlation between the deficiency of omega-3 fatty acids with an increase in rational deterioration, Alzheimer's and Parkinson's (Parrott & Greenwood, 2007) [16]. The presence of trace elements such as zinc and copper in the diet is critical to the development of neurotransmitters, energy metabolism, and antioxidant defense of the brain (Jones *et al*. 2008) [14]. The administration of carob extract to mice has reduced depression (Agrawal *et al*. 2011) [1].

Custódiio *et al*. reported that carob leaf extract inhibits the proliferation of breast cancer cells by stimulating apoptosis (Custódio *et al*. 2008) [8]. Other researchers reported that the carob plant prevents the proliferation of liver cancer cells in the culture. In addition, it induces apoptosis and activates caspase 3 (Corsi, 2002) [4].

Klenow and colleagues reported that carob extract in culture via apoptosis stimulation prevent colon cancer cell proliferation (Klenow *et al*. 2008) [13]. Similar results were taken by Ghanemi and colleagues for this herbal medicine (Ghanemi *et al*. 2017) [10]. Inconsistent with these researches, in the present study carob administration does not change the number of dark neuron in hippocampus compared to the sham group. It seems that the above studies investigated the anti- cancer effect of the carob extract, and considering that in the present study was not observed significance difference in the number of dark neuron between the sham group and experimental groups, it may be concluded that this plant does not have toxic effects on healthy cells**.**

Based on our search, there is no study on the effects of administration of carob on brain, so far. In this study, chloroform was used for deep anesthesia, which it was better than replaced with ether. In staining with Toluidian blue, the neurons observe very picnotic, small and dark that it may be mistake with neuroglia. As a result, the number of neurons exceeds from the normal rate. Galia staining is a better choice for dark neurons, and it eliminates this problem.

In this study, it was better that apoptosis investigate with Tunnel Kit. In addition, his to pathologic evaluation the hippocampus recommend to dear researchers. The effects of administration this plant on the other of the parts of the brain as well as cerebellum using crisel violet or galia staining is also recommended. The results of this study showed that administration of 200-800 mg/kg carob extract to adult male mice does not change the number of dark neurons of the hippocampus of the adult mice compared to the sham group.

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