RESEARCH PAPER

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The effects of bee venom on behavior and the role of leptin in rats

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The aim of this study is to evaluate the dose-dependent effect of bee venom (BV) on behavioral functions in rats and the physiological role of leptin in the prefrontal cortex, hippocampus, and amygdala tissues. Adult Sprague-Dawley male rats were used in the experiments. The rats were divided into three groups of control, 0.1 mg/kg BV, and 0.5 mg/kg BV. The rats were injected with BV subcutaneously for 15 consecutive days. The open field test (OFT), the elevated plus maze test (EPM), and the forced swimming test (FST) were performed as behavioral assessments. Animals were sacrificed, and brain regions were removed. Leptin levels were measured in various brain regions by ELISA. In the OFT, the total distance and speed for the 0.1 mg/kg BV group increased compared to controls and the 0.5 mg/kg BV group. In the EPM, the 0.1 mg/kg BV group remained in the open arm for a significantly longer period of time compared to the other groups. In the FST, the 0.5 mg/kg BV group was more mobile than the other groups. Leptin levels in the prefrontal cortex were significantly higher in the 0.1 mg/kg BV group compared to the control and 0.5 mg/kg groups. There were no significant differences between groups in hippocampus and amygdala leptin levels. The results of the study show that BV has a positive effect on behavioral parameters. BV may have a positive effect on anxiety- and depression-like behaviors by increasing leptin levels in the prefrontal cortex.

Key words: bee venom, leptin, anxiety-like behavior, prefrontal cortex

INTRODUCTION

Anxiety is considered a normal part of life and can arise in relation to different situations. Fear and anxiety-related behaviors are actually adaptive behaviors that serve to protect the organism. Anxiety arises when a potential danger is perceived, allowing the organism to continue its life by protecting itself from the dangerous situation. If these behaviors are persistent, excessive or cannot be corrected clinically, they are defined as a mental disorder (Mahan and Ressler, 2011). All anxiety disorders are associated with feelings of anxiety and physiological stress reactions such as tachycardia, hypertension, nausea, respiratory distress, sleep disturbances and high glucocorticoid levels (Pinel and Barnes, 2018).

The prefrontal cortex (PFC), hippocampus, and amygdala play a role in both depression and anxiety disorders. Most of the evidence linking these structures to anxiety disorders comes from functional neuroimaging studies, where atypical activity in these regions is recorded during the performance of various emotional tasks. The PFC is connected to regions related to emotional behavior (Kim and Whalen, 2009; Nitschke et al., 2009). A lesion in the PFC can cause depression- and anxiety-like behaviors in animal models (Lacroix et al., 1998; Klein et al., 2010).

Leptin, produced by the obese gene as a 16-kDa protein, is a hormone that is released into blood vessels and crosses the blood-brain barrier with the help of a selective carrier (Banks et al., 1996). Studies have shown that leptin is effective in controlling energy

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balance and regulating other physiological processes such as reproduction and cognition (Chehab, 2000; Farr et al., 2006). Leptin acts in the regulation of energy homeostasis by interacting with receptors in specific hypothalamic nuclei. Leptin has six different receptors; its long form that is capable of signaling from these receptors is widely distributed in brain regions involved in emotional responses, such as the hippocampus (Elmquist et al., 1998; Ahiama and Osei, 2014). The signals produced by leptin and its receptors have important functions in the modulation of cognitive processes, neurogenesis, synaptogenesis, and neuroprotection (Dodd et al., 2013). A preclinical study in rodents demonstrated the antidepressant effects of leptin in a model of depression caused by chronic stress (Lu et al., 2006). Increases in circulating leptin paired with increases in body weight and anxiety-like behaviors support the mechanism that high-fat diets can induce anxiety and insensitivity to leptin (Morton, 2011).

Bee venom (BV) and its ingredients have recently begun to be used widely in traditional and complementary medicine, cosmetics and new drug development. BV consists of a complex mixture of polypeptides, enzymes, lipids and bioactive amines (Dennis et al., 2011). Studies with bee venom have shown that BV has anti-inflammatory, anti-rheumatoid, and analgesic effects (Lee et al., 2005). It has been shown that BV prevents the migration of cancer cells and cell differentiation induced by obesity and neurodegenerative diseases (Silva et al., 2015; Cheon et al., 2017). In a study by Florea et al. (2011), functional changes and neuronal dysfunctions produced experimentally in the cerebral cortex after subcutaneous BV injections were investigated. The results showed that BV had a dose-dependent effect on the nervous system. Low-dose BV was a very potent neurostimulator, but high-dose BV had a pronounced neurotoxic effect that was visible by EEG after 15 min (Florea et al., 2011). Previous studies have shown that bee venom has effects on the brain, but whether bee venom affects behavior in rats has not been investigated.

The aim of this study is to evaluate the dose-dependent effect of BV on behavior, as well as leptin levels in the PFC, hippocampus and amygdala, in rats.

METHODS

Animals

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Ethics Committee of the Dokuz Eylul University Medical Faculty, Turkey (approval number: 61/2019). All experimental procedures met the standards of the Guide for the U.S. Public Health Service and NIH regarding the care and use of animals for experimentation.

Rats were housed individually in a polycarbonate cage, at a room temperature of 20-22°C, under a 12 h light-dark cycle. The rats had free access to food and water. Twenty-one male Sprague-Dawley rats, ten weeks of age, weighing 200 (±30) g were used. Bee venom (whole venom of *Apis mellifera*; BV) was supplied by Zafer Egeli, a member of the Denizli Beekeepers Association (Turkey) and was stored at -20°C. The BV was dissolved in 0.5 mL of physiological saline before use. The treatment with BV for 15 days did not cause injury to the animals.

Experimental design

The rats were randomly allocated to 3 study groups (n=7) of Group I: Control; Group II: 0.1 mg/kg BV, where rats were injected with 0.1 mg/kg BV subcutaneously for 15 days (0.1 BV) (Lee et al., 2010); and Group III: 0.5 mg/kg BV, where rats were injected with 0.5 mg/kg BV subcutaneously for 15 days (0.5 BV) (Mousavi et al., 2012).

On the 15th day, the rats underwent the behavioral open field test (OFT), the elevated plus maze test (EPM) and the forced swimming test (FST). Animals were euthanized on the 16th day, and brain regions were taken. Leptin levels were measured in the PFC, hippocampus, and amygdala tissues using the enzyme-linked immunosorbent assay (ELISA) method (Fig. 1).

Behavioral tests

Behavioral tests were performed on the last day of injections. The animals were brought into the room at least 30 min before the start of the behavioral tests to acclimate to the test environment. All the behavioral tests were recorded and analyzed by the Noldus Ethovision XT video-tracking system.

Open Field Test (OFT)

When an experimental animal is left in an environment different from its own, an increase in locomotor movements can be observed, along with an increase in unrest. Therefore, the OFT was used for assessing anxiety. For the OFT, a 1 m × 1 m × 50 cm

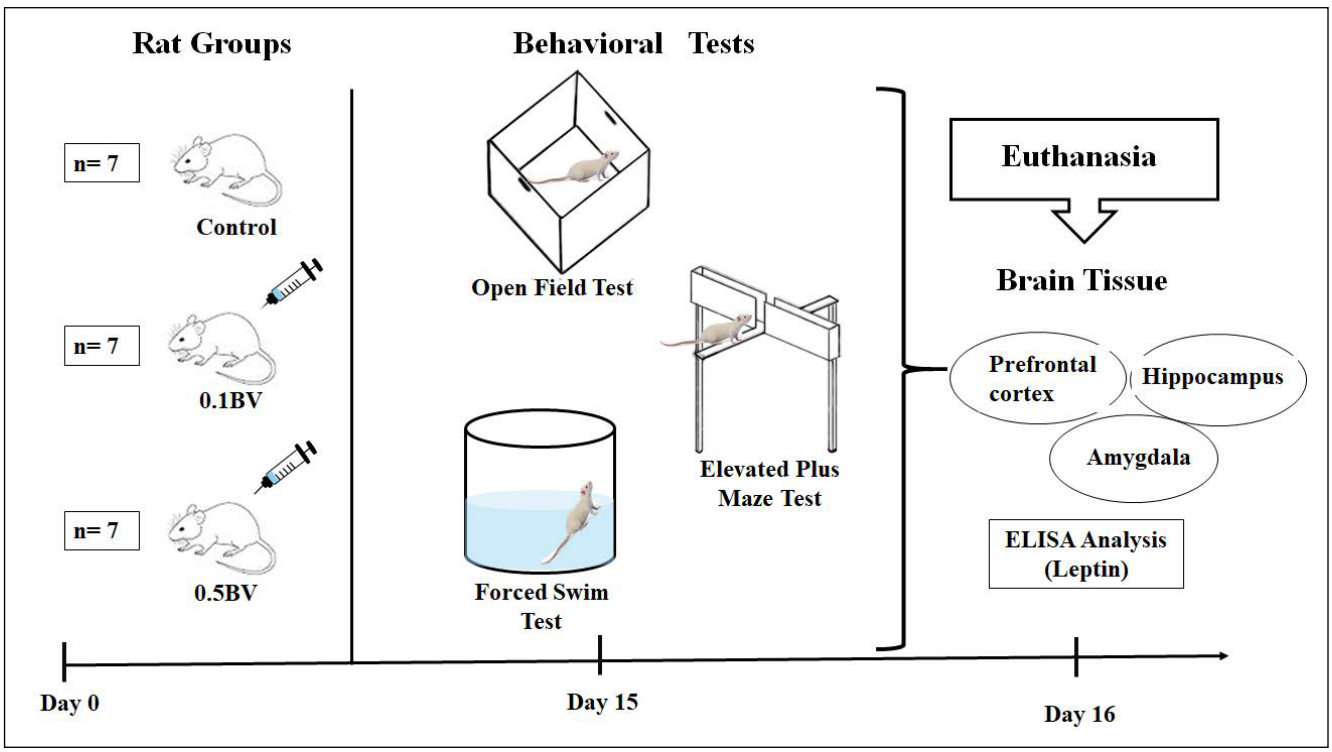

Fig. 1. The scheme of the experiment.

floor was illuminated with a 100 lux light source. The experimental animal was placed in the middle of the area, and its movements were recorded and analyzed during the 5-min test. The ground was divided into 16 squares (12 on the edges, 75%; 4 in the center, 25%). The system automatically recorded the time spent at the edges of the field and in the middle of the field and the total distance traveled in the field (Gokdemir et al., 2020).

Elevated Plus Maze Test (EPM)

When rats are left in an unfamiliar environment, an elevated floor path can increase anxiety. The EPM was used to evaluate anxiety-like behavior. The maze was elevated 50 cm above the floor and consisted of a central platform (5 cm, L × 5 cm, W) with two open arms (50 cm, L × 10 cm, W × 0.5 cm, H) and two closed arms (50 cm, L × 10 cm, W × 40 cm, H). The experimental animal, placed in the middle of this EPM, was analyzed by its recorded movements during a 5-min test period. During this time, the time spent in open and closed arms was recorded. Accordingly, the time spent in the closed arms and open arms is considered proportional to high- and low-level anxiety responses, respectively (Gokdemir et al., 2020).

Forced Swim Test (FST, Porsolt Test)

The FST was used for assessing depression-like behavior. Each rat was placed in a water-filled clear Plexiglas cylinder (50 cm, H × 30 cm, diameter). It was filled with 25°C water to a depth of 20-25 cm to force the animals to swim and prevent them from jumping. The total distance traveled, and the duration of immobility was recorded for 5 min (Gokdemir et al., 2020).

Measurement of leptin in brain tissues

The animals were euthanized 24 h after the last behavioral experiment. Brain tissue samples were removed for biochemical measurements. The PFC, hippocampus and amygdala regions, which are related to anxiety, were dissected on ice. The level of leptin protein in the tissues was measured by ELISA using a plate reader according to the manufacturer's instructions (BioTek, Vermont, USA, detection range: 0.16-10 ng/mL). Leptin levels were measured using the Rat Leptin ELISA Kit (Elabscience, Houston, Texas, United States). For the measurement of tissue protein levels, the Pierce BCA Protein Assay Kit (Thermo Scientific, Illinois, USA) was used.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Differences between the groups were assessed with one-way ANOVA with a *post-hoc* LSD test using SPSS 22.0 (IBM Inc., IL, USA). Correlation analysis was performed using the Pearson correlation test. $P<0.05$ was considered significant.

RESULTS

In the OFT, the total distance traveled increased in 0.1 BV rats compared to controls and 0.5 BV rats ($F_{2,18}=12.55$, $P<0.001$; Fig. 2A). The time spent in the periphery of the OFT in the 0.1 BV group was significantly less than in the other groups ($F_{2,17}=5.87$, $P=0.012$; Fig. 2B). There was no significant difference found for the time spent in the center ($P>0.05$; Fig. 2C).

In the EPM, the 0.1 BV rats' anxiety levels were lower than in the other groups. The total distance traveled was higher in the 0.1 BV rat group compared to controls and the 0.5 BV rat group ($F_{2,18}=6.37$, $P=0.008$; Fig. 3A). The 0.1 BV group remained in the open arm for a significantly longer time compared to other groups, ($F_{2,17}=5.81$, $P=0.012$; Fig. 3B). Although the 0.1 BV rats remained in the closed arm for less time than the other groups, the difference was not statistically significant, ($P>0.05$; Fig. 3C).

In the FST, there were no significant differences in the total distances traveled among the three groups ($P>0.05$). However, the 0.5 BV group rats displayed less immobility than the other groups ($F_{2,18}=35.24$, $P<0.001$; Fig. 4A, B).

Leptin levels in the PFC were significantly higher in the 0.1 BV group compared to the control and 0.5 BV groups ($F_{2,18}=4.93$, $P=0.02$). There were no significant differences between groups in hippocampus and amygdala leptin levels ($P>0.05$, Table 1). The levels of leptin in the PFC strongly positively correlated with time spent in the open arms of EPM ($r=0.518$, $P=0.016$).

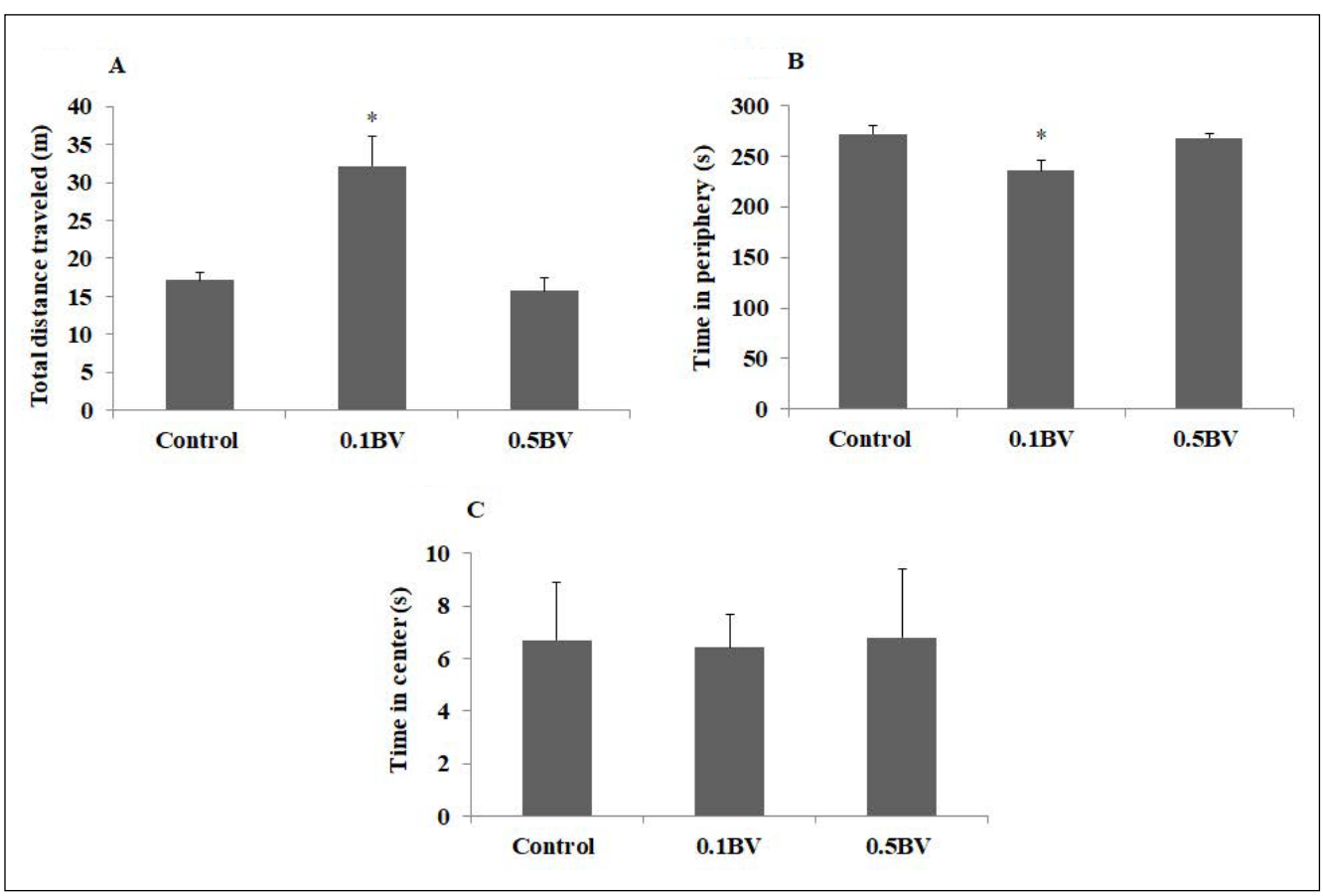

Fig. 2. The open field test results. (A) Total time traveled was increased in 0.1 BV rats compared to control and 0.5 BV rats (*$P<0.05$). (B) The time spent in the periphery for the 0.1 BV group was significantly less than the other groups (*$P<0.05$). (C) There were no significant differences for time spent in the center.

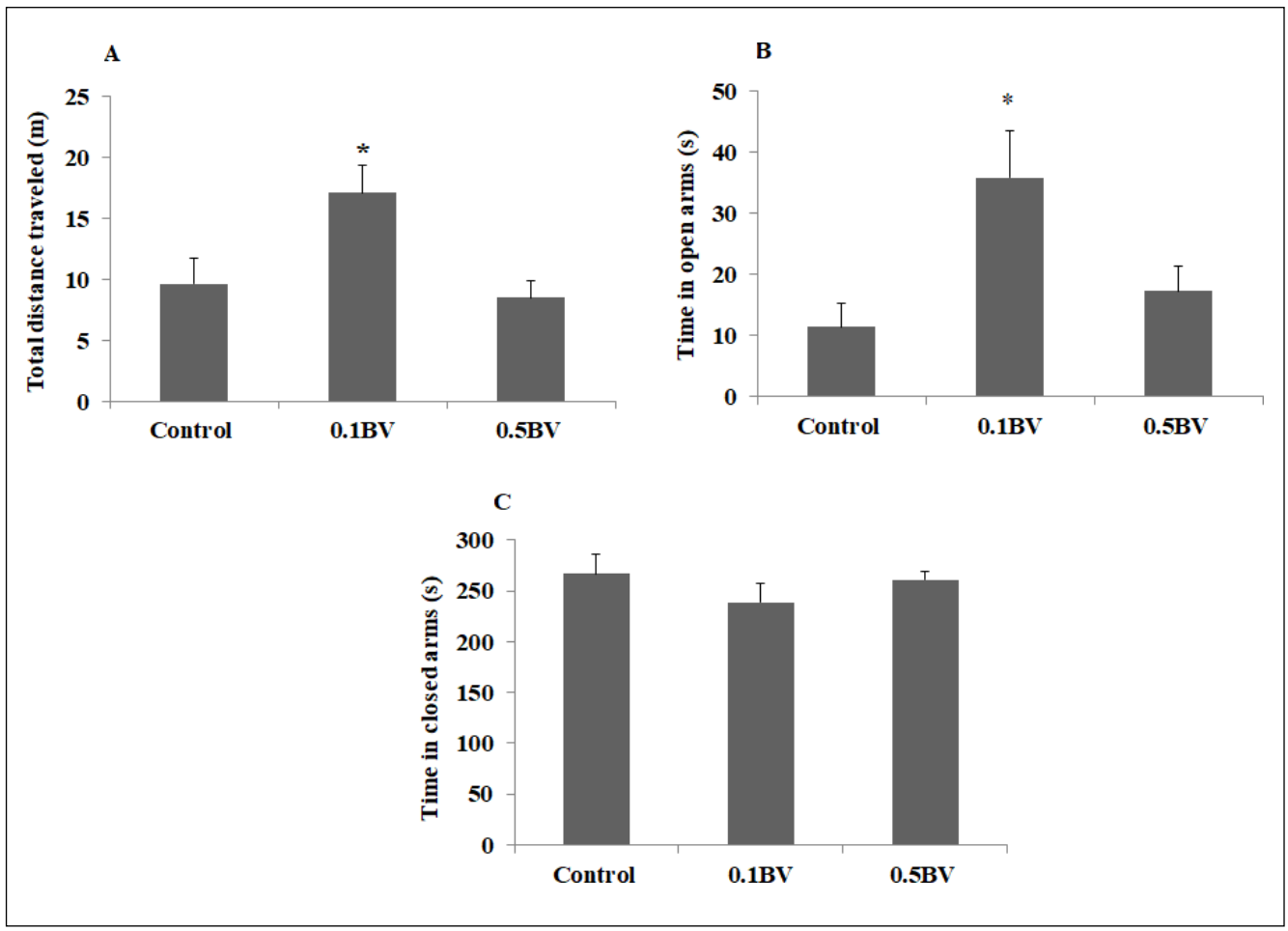

Fig. 3. The elevated plus maze test results. (A) Total distance traveled increased in the 0.1 BV rats compared to controls and 0.5 BV rats (*P<0.05). (B) The 0.1 BV group remained in the open arm for a significantly longer period of time compared to other groups (*P<0.05). (C) There was no significant difference between groups for time spent in the closed arm.

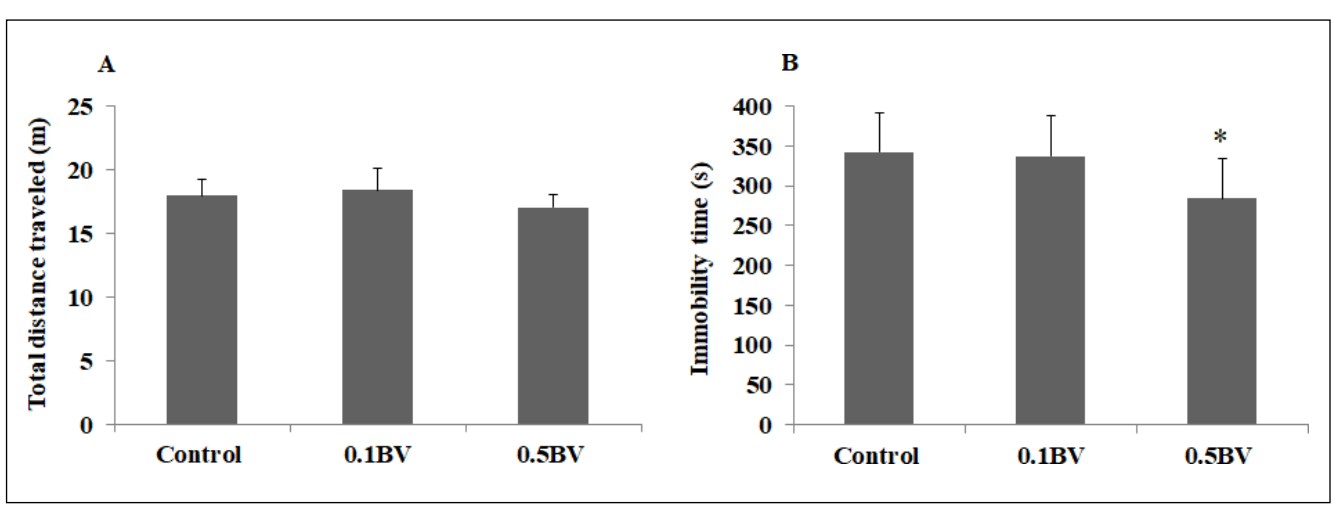

Fig. 4. The forced swim test results. (A) No significant differences were found between groups for total distance. (B) The 0.5 BV rats displayed less immobility than the other groups (P<0.05).

Table 1. Leptin levels (pg/mg) in rat brain areas.

Groups	Prefrontal Cortex	Hippocampus	Amygdala
Control	0.53 ± 0.03	0.58 ± 0.05	0.20 ± 0.06
0.1 BV	0.77 ± 0.1*	0.45 ± 0.02	0.20 ± 0.06
0.5 BV	0.45 ± 0.03	0.63 ± 0.2	0.25 ± 0.07
p (Control *vs.* 0.1 BV)	0.035	0.350	0.590
p (Control *vs.* 0.5 BV)	0.476	0.719	0.489
p (0.1 BV *vs.* 0.5 BV)	0.008	0.202	0.226

Data are expressed as mean ± SEM, n=7/group. BV: bee venom, SEM: standard error of the Mean, and * denotes a significant difference relative to control and 0.5 BV groups.

DISCUSSION

In the present study, we showed that the administration of BV changed the behavioral phenotype of animals by decreasing anxiety and depressive-like behaviors, and it also increased leptin levels in the rat PFC. Our results suggest that BV may be associated with leptin levels in PFC and that BV may have a positive effect on rat behavior at certain doses.

In many experimental studies, the PFC has been shown to affect the amygdala and control stress and emotional responses. In humans, it has been shown that ventromedial PFC activity is associated with anxiety and reduces anxiety when it works properly (Kim and Whalen, 2009). Individuals with PFC damage have been shown to experience problems in emotional regulation both in their daily life and in anxiety tests (Anderson et al., 2006).

Anxiety behavior in an animal left in an open area is triggered by two factors: the animal being taken from its own environment and left alone in an unfamiliar en-vironment and the fear of wide space, called agorapho-bia. Open and wide environments are sources of stress for rodents (Prut and Belzung, 2003). It is important to note whether an animal in the open field setup trav-els more on the sides or in the center of the chamber. An animal removed from its familiar environment will avoid going into the middle of the area and will move less. In the OFT, the fact that total movement distance was higher and that time spent in the periphery was less in the 0.1 BV group compared to control group shows that this dose of BV is associated with decreased anxiety in rats.

The EPM test measures the time taken for an animal placed in a closed elevated arm to enter the open elevated arm and the time spent in this arm. It has been shown that anxiolytic agents increase the time for both of these measures, while anxiogenic agents decrease the time (Rodgers et al., 1997; Ferguson et al., 2004). The 0.1 BV group rats remained in the open arm for a significantly longer period of time compared to the other groups, which demonstrated less anxiety in this group. For time spent in the closed arm, no significant difference between groups was found.

The FST has been shown to have high predictive validity for antidepressant activity. In this test, animals show "hopelessness" behavior as well as showing escape behaviors such as inactivity, swimming, and climbing (Lucki, 1997). High doses of BV were associated with decreased immobility and increased swimming time. These results show that administration of high doses of BV may result in antidepressant activity.

Leptin also plays a role in enhancing transmission by increasing the excitability of neurons (Harvey, 2007) and increasing synaptic motility and density, particularly in hippocampal neurons (O'Malley et al., 2007). Leptin is generally neuroprotective and has generated interest as a potential antidepressant and as a neuroprotective agent in Alzheimer's disease (Dietrich et al., 2008). Leptin levels in the PFC were significantly higher in the 0.1 BV group compared to the control and 0.5 BV groups; high leptin levels in the PFC may have caused the lower levels of anxiety that were observed in this group. Studies have shown that there may be a correlation between PFC leptin levels and anxiety-like behaviors (Ates et al., 2014). We also observed a strong positive correlation between time spent in the open arms and PFC leptin levels in the 0.1 BV group. In line with the literature, we suggest that there may be a correlation between anxiety-like behavior and leptin levels.

CONCLUSION

BV can affect a rat's ability to control emotional behavior by acting through the PFC *via* leptin. Further studies are needed to elucidate the relationship between BV, leptin and anxiety.

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