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Chlorogenic acid, gallic acid and ferulic acid prevent the development of hyperactivity and anxiety in olfactory bulbectomized rats

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Chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA) are abundant biologically active polyphenols in human diet. The aim of this study was to investigate the effects of CGA, GA and FA on the behavior of rats subjected to bilateral olfactory bulbectomy (OB) using the elevated plus maze test. Experimental rats were divided into 5 groups (n=6): sham operated (SO), OB, OB+CGA, OB+GA and OB+FA. After a 15-day recovery period after the operation, rats were treated orally in the course of 14 days. SO and OB rats received saline, OB+CGA, OB+GA and OB+FA groups were treated with CGA, GA and FA (20 mg/kg), respectively. OB induced a state of hyperactivity and anxiety. CGA, GA and FA antagonized the behavioral changes induced by OB. GA and FA caused restoration of the measured indices to values that were significantly different from those of OB rats and did not differ from those of SO rats. The effect of CGA was even higher. It increased the open arms entries and open arms time, as well as the ratios open arms entries/total arms entries and open arms time/total arms time to values that were significantly higher not only from those of OB rats but also from those of SO rats. Similarly, the closed arms time of OB+CGA rats was lower than the respective time of both OB and SO rats. In conclusion, CGA, GA and FA prevented the development of hyperactivity and anxiety in OB rats. Most pronounced was the effect of CGA.

Keywords: chlorogenic acid, gallic acid, ferulic acid, hyperactivity, anxiety, olfactory bulbectomized rats

INTRODUCTION

Anxiety disorders are widespread psychiatric problems affecting human society [1]. They are often associated with depressive conditions or other mood disorders [2-5], and chronic illnesses [3]. The conventional treatment is accompanied by various side effects [6]. Natural products have been considered an alternative option for the treatment of these disorders with conceivably minimized adverse effects, and/or innovative mechanisms of action [7]. Plant polyphenols represent promising agents for treatment of central nervous system (CNS) diseases [8].

Phenolic acids are polyphenolic compounds of natural origin. Chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA) are widespread biologically active phenolic acids in fruits, vegetables, nuts, coffee and tea, wine, whole grains [9-12]. It has been reported that CGA or its metabolites may cross the blood-brain barrier and exert neuroprotective effects on brain tissue [13, 14]. FA was found in rat brain approximately thirty min after its oral administration [15]. Some experiments have demonstrated an anxiolytic-like effect of CGA in mice [16]. FA and CGA have shown neuroprotective and cognition-enhancing effects in models of Alzheimer's disease [17-19]. In the study of Han *et al*., FA stimulated neural progenitor cell proliferation *in vitro* and *in vivo* [20]. GA treatment against trimethyltin-induced hippocampal degeneration ameliorated the depression-anxiety state in rats [21]. GA has been found to exert neuroprotective effects on amyloid β-mediated neurotoxicity [22].

The olfactory system in the rat forms a part of the limbic region, in which the amygdala and hippocampus contribute to the emotional and memory components of behavior. Bilateral removal of the olfactory bulbs in rodents induces behavioral deficits that reflect a dysfunction of the cortical-hippocampal-amygdala circuit. Olfactory bulbectomy (OB) in rats is associated with a variety of behavioral abnormalities and serves as a model of depression with comorbid anxiety, agitation, sexual and cognitive dysfunction [23-26]. Surgical removal of olfactory bulbs in experimental rodents is considered most suitable for studying the neurochemical mechanisms underlying the pathophysiology of these behavioral disorders.

Taking into consideration the above mentioned data, the aim of this study was to investigate the effects of CGA, GA and FA on the behavior of rats subjected to bilateral OB using the elevated plus maze test.

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EXPERIMENTAL

Animals and experimental substances

Male Wistar rats (weighing 200-220g) were used in this study. Experimental rats were divided into 5 groups of 6 animals each: sham operated (SO), OB, OB+CGA, OB+GA and OB+FA. They were housed in polypropylene boxes under a normal 12 h light to 12 h dark schedule (lights on at 6:00 am). All rats had free access to food and drinking water. Ambient temperature was maintained at 22-25°C. Animals were allowed to adjust to the housing conditions before experiments began. The behavioral test was conducted between 10:00 am and 1:00 pm. After the testing procedure, the rats were returned to their respective home cages.

All procedures concerning animal treatment and experimentation were conducted in compliance with the national laws and policies, in conformity with the international guidelines (EU Directive 2010/63/EU for animal experiments).

CGA, GA and FA were purchased from Sigma-Aldrich, Germany.

Surgical procedure: Bilateral olfactory bulbectomy (OB)

Bilateral OB was performed according to the method, described by Kelly et al. [23]. Rats were anesthetized (with intraperitoneal injections of Calypsol 50 mg/kg) and placed in a stereotaxic apparatus (Stoelting Co, USA). The coordinates of the olfactory bulbs were determined according to the stereotaxic atlas of Pellegrino and Cushman [27]. The head was shaven and 1.0 cm midline scalp sagittal incision was made. Then bilateral 2.0 mm burr holes were drilled (8.0 mm anterior to bregma and 2.0 mm from the midline). The bulbs were aspirated with a stainless needle attached to a water pump. The burr holes were then plugged with a hemostatic sponge (Gelaspon) to control the bleeding after the drilling.

After the surgery animals were treated daily with antibiotics – topically (with Nemybacin for 7 days) and intraperitoneally (with Gentamicin for 5 days). After the OB procedure, the rats were housed in groups of two and were handled daily during a 15-day recovery period. SO rats are treated similarly, except that the olfactory bulbs were left intact.

Verification: The extent of the lesion was assessed visually post-mortem.

Animal treatment

After a 15-day recovery period, rats were treated orally in the course of 14 days. SO and OB rats received saline (10 ml/kg), OB+CGA, OB+GA and OB+FA groups were treated respectively with CGA, GA and FA (20 mg/kg as a 10 ml/kg solution).

Behavioral experiment: Elevated plus maze (EPM) test

On the 14th day, 60 min after the last treatment, the animals were tested in the EPM, a frequently used test for studying anxiety in rodents and the anxiolytic activity of new drugs [28]. The EPM consisted of four arms, 50 cm long and 10 cm wide, elevated 50 cm above the ground. The apparatus was illuminated by a 40 W bulb positioned 50 cm above it.

Each rat was placed in the center of the maze facing one of the open arms. An arm entry was counted when the animal placed all four paws into the arm. The indices recorded during the 5-min test period were: number of entries into the open arms and time spent there, number of entries into the closed arms and time spent there, total number of arms entries, the ratio: number of open arms entries vs. total number of arms entries and the ratio: open arms time vs. total time in the arms. An increase in the number of entries into the open arms and the time spent there is regarded as a powerful marker for the anxiolytic effect of the tested substance [28,29]. After each assay, the EPM was carefully cleaned with 70% ethyl alcohol solution and dried to remove olfactory cues.

Statistical analysis

All analyses were performed using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using the Students's *t*-test. All results are expressed as mean±S.E.M. A level of $p < 0.05$ was considered significant.

RESULTS

OB induced a state of hyperactivity demonstrated by a significant [$p < 0.001$] increase in the total number of arms entries of OB rats in comparison with SO rats (Fig. 1). The changes in the other indices demonstrated the development of a state of anxiety. Compared to SO rats, OB animals had a significantly lower number of entries into the open arms [$p < 0.05$] (Fig. 2A) and time spent there [$p < 0.01$] (Fig. 3A), significantly higher number of entries into the closed arms [$p < 0.001$] (Fig. 2B) and closed arms time [$p < 0.01$] (Fig. 3B), as well as significantly lower ratios open/total arms entries [$p < 0.001$] (Fig. 4A) and open/total arms time [$p < 0.01$] (Fig.4B).

Treatment of OB rats with CGA, GA and FA antagonized the behavioral changes induced by OB.

GA and FA caused restoration of the measured indices to values that were significantly different from those of OB rats and did not differ from those of SO rats (Figs. 1, 2 and 3). The effect of CGA was even higher. It increased the number of entries into the open arms and time spent there [$p < 0.001$] (Fig. 2A, 3A), as well as the ratios open arms entries/total entries [$p < 0.001$] (Fig. 4A) and open arms time/total time in the arms to values that were significantly higher [$p < 0.001$] (Fig. 4B) not only from those of OB rats but also from those of SO rats. Similarly, the closed arms time of OB+CGA rats was lower [$p < 0.001$] (Fig. 3B) than the respective time of both OB and SO rats.

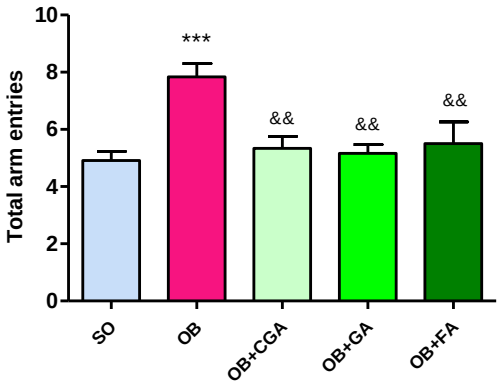

Fig. 1. Total number of arms entries in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&}p<0.01$, *vs.* olfactory bulbectomized (OB)

DISCUSSION

Removal of the olfactory bulbs in rats causes structural and functional alterations in brain regions that result in behavioral changes including anxiety-resembling behavior [24], exploratory hyperactivity, depressive mood, and irritability [30].

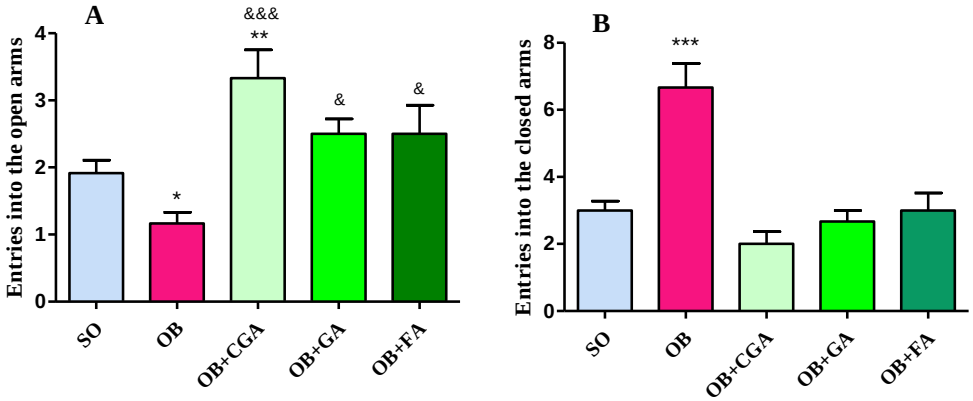

Fig. 2. Number of entries into the open arms (**A)** and number of entries into the closed arms (**B)** in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&}<0.05$, $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

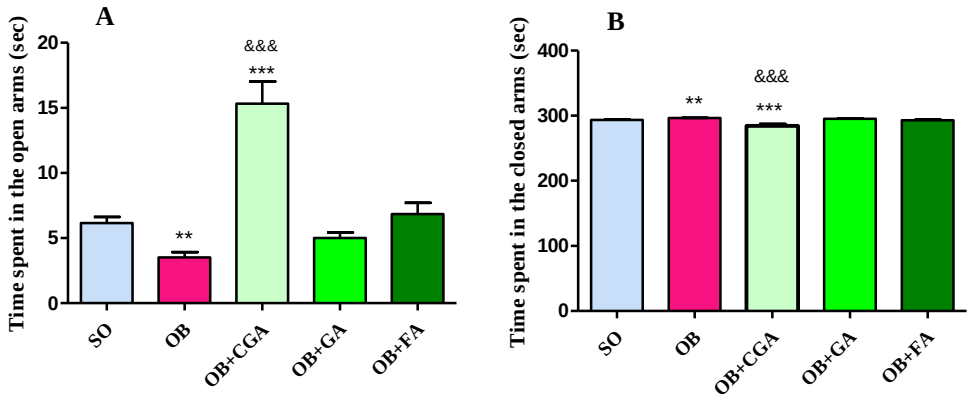

Fig. 3. Time spent in the open arms (sec) (**A)** and time spent in the closed arms (sec) (**B)** in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

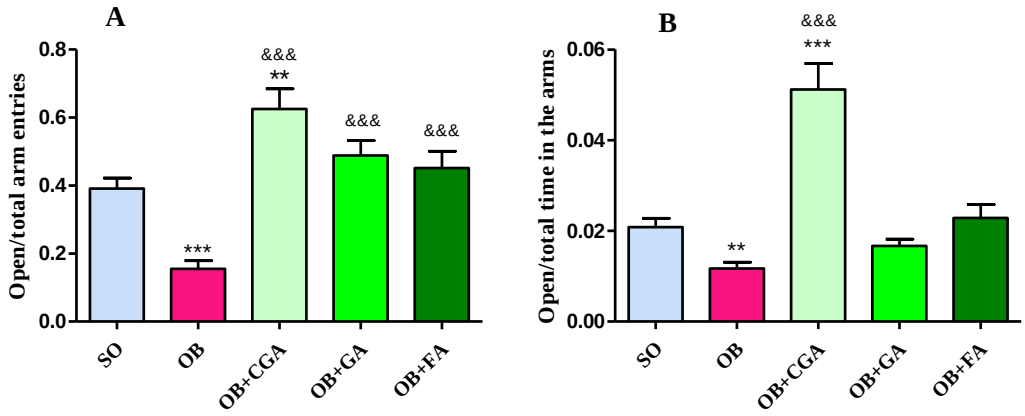

Fig. 4. Ratio of open arms entries vs. total entries in the arms (**A**) and ratio of open arms time vs. total time in the arms (**B**) in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

Bilateral olfactory increases the levels of reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), and decreases glutathione and brain-derived neurotrophic factor (BDNF) in mammalian hippocampus [24,31,32].

In this experiment, OB resulted in hyperactivity demonstrated by the increase of the total number of arms entries. In other studies, such behavioral hyperactivity in OB rats was related to the increased glutamate level in the *striatum* [33] and *nucleus accumbens* [34]. In the present experiment, CGA, GA, FA decreased the hyperactivity of OB rats. Mikami at al. revealed that CGA reversed the glutamate-induced toxicity, as well as glutamate-induced death of primary cells isolated from mouse cortical neurons [35]. In another study, CGA and its metabolites reversed the glutamate-induced toxicity in primary cultures of rat cerebellar granule neurons [36].

Excessive glutamate concentration can induce oxidative stress by increasing the production of ROS, strongly related to the pathogenesis of anxiety behaviors. Literature data show that the pathophysiology of anxiety and related affective disorders is associated with a wide range of epigenetic changes: increased oxidative stress [37, 38], neuroinflammation [39], glutamatergic dysfunction [40], dysregulation of synaptic plasticity through alterations at the neurotrophin level and inhibition of signaling pathways [41]. The implication of oxidative stress in the pathogenesis of anxiety disorders (obsessive-compulsive disorder and panic disorder) was also suggested by Kuloglu *et al.* [42]. Another mechanism that might contribute to the pathogenesis of anxiety is the low level of BDNF [43]. Rinwa *et al*. revealed elevated levels of inflammatory cytokines (TNF-α) and caspase-3 accompanied by a marked reduction in BDNF in the brain of OB rats [44].

In this experiment, CGA, GA, FA showed an anxiolytic-like effect. Most of the biological actions of phenolic acids on the brain have been attributed to theirs anti-inflammatory and antioxidant properties [45]. A study of Gul *et al.* revealed a neuroprotective effect of CGA. That polyphenol attenuated the H_2O_2-induced increases in the levels of malondialdehyde and ROS in rat cortical slices [46]. Another experiment on primary cultures of rat cerebellar granule neurons revealed that CGA increased the protection against H_2O_2-induced proteasome inhibition and caspase-dependent intrinsic apoptosis [47]. In a study of Moghadas *et al.*, the mood stabilizing and neuroprotective effects of GA were attributed to the anti-oxidant activity and amelioration of cell density loss in the hippocampus [21]. Lenzi *et al.* showed that the effects of FA on the CNS were also coupled with its antioxidant activity, evidenced by increased superoxide dismutase and catalase activities, as well as low thiobarbituric acid reactive substances levels, found in hippocampus of treated mice [48]. In the study of Liu *et al*., FA increased the levels of BDNF in the prefrontal cortex and hippocampus, as well as inhibited microglia activation, pro-inflammatory cytokines expression, nuclear factor kappa B signaling [49]. The reduction of pro-inflammatory cytokines could contribute to the anxiolytic-like effects of phenolic acids. FA significantly inhibited the production of the TNF-α, IL-6, IL-1β, and NO, and reduced COX-2 and iNOS [50].

Receptor activation might also participate in the anxiolytic-like effects of phenolic acids. Bouayed *et al*. demonstrated an anxiolytic effect of CGA in mice

tested by EPM, light-dark test and free exploratory test [51]. In that study, the anxiolytic-like effect of CGA was reversed by the benzodiazepine antagonist flumazenil, and the authors suggested that CGA might act as a benzodiazepine receptor agonist [51]. 5-HT1A receptors are involved in the modulation of exploratory and fear-related behaviors, and reductions in 5-HT1A receptor density resulted in increased anxiety [52]. In the EPM tested rats, Mansouri and colleagues observed an anxiolytic-like activity of GA similar to the 5-HT1A receptor agonist buspiron [53].

In conclusion, chlorogenic acid, gallic acid and ferulic acid prevented the development of the state of hyperactivity and anxiety in olfactory bulbectomized rats. Most pronounced was the effect of chlorogenic acid.

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