Original Article

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Exploring the impact of endogenous AHR ligand, 6-formylindole [3,2-b]carbazole (FICZ) on the microbiota-gut-brain axis in chronic stress-induced depression in rats

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Abstract

Aryl hydrocarbon receptor (AHR) is a nuclear receptor that mediates responses to environmental stimuli. The microbiome, influenced by factors such as nutrition, antibiotics, stress, and infection, plays a crucial role in mood, cognition, and mental health through the gut-brain axis. Chronic stress, known to impact both mental and physical health, is associated with depression onset, with social defeat being a potent stressor. This study investigates the effects of endogenous AHR ligands on the microbiota-gut-brain axis and behavioral changes in rats subjected to chronic stress. Male rats were divided into six groups: control, social defeat (SD), treatment with endogenous AHR ligand 6-formylindole [3,2-b]carbazole (FICZ) ± SD and treatment with tryptophan (TRP) ± SD. AHR ligands were administered weekly for one month, and fecal samples were collected for microbial analysis. Forced swimming and splash tests were used to assess depression behaviors. The results showed FICZ positively impacting depression-like behaviors, while chronic stress and depression correlated with decreased Lactobacillus species frequency, especially in the social defeat group. Further investigations are warranted to explore the impact of social defeat stress on microbial populations across other groups and other bacterial species.

Keywords: AHR, 6-formylindole [3,2-b]carbazole (FICZ), Tryptophan, Microbiota-Gut-Brain-Axis, Social defeat, Depression

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1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor regulating genes involved in xenobiotic metabolism, including cytochrome P450 isoforms 1A and 1B (1-4). AHR exhibits promiscuity in binding various ligands (5-7), with endogenous ligands deriving from the tryptophan pathway, such as 6-formylindole [3,2-b]carbazole (FICZ), exerting significant effects (3, 7). AHR, expressed in multiple organs and tissues, plays a crucial role in the microbiome-gut-brain axis (8, 9), as demonstrated in various animal models (10). The microbiome interacts with the brain through multiple pathways, including the immune system, tryptophan metabolism, and the vagus and enteric nervous systems. Stress, a key influencer of microbiota composition, affects the microbiota-gut-brain axis across all life stages, with implications for mental health (11). Gut microbiota plays a crucial role in how the body exerts an impact on the brain, contributes to maintaining a state of normal and healthy balance within the body, and influences the likelihood of developing

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various diseases, such as anxiety and mood disorders. An indirect role of microbiota in the stress response has recently been demonstrated in animal stress models. It is known that stress increases intestinal permeability, allowing bacteria to cross the intestinal mucosa and directly reach the neuronal cells of the immune system and enteric nervous systems (ENS) (12). The gut microbiota secretes neurotransmitters and neuromodulators such as gamma-aminobutyric acid (GABA), serotonin, catecholamines and acetylcholine. These neurotransmitters can affect the host's brain and behavior by causing epithelial cells to release molecules that regulate nerve signals in the ENS (13). Depression, a complex disorder involving factors like chronic stress, neuro-inflammation, and neurotransmitter dys-regulation, is closely linked to tryptophan metabolism and microbiota activity (14). The synthesis of kynurenine (KYN) from L-tryptophan is affected by many different factors, including inflammatory processes. KYN and its metabolic activities are linked with diverse neuropsychiatric conditions, such as major depressive disorder (MDD) and bipolar disorder (15).

The aim of this study is to investigate how exposure to AHR ligands influences the microbiota-gut-brain axis and its potential role in depression onset in rats.

2. Material and methods

2.1. Chemicals

Chemicals including FICZ and tryptophan were obtained from reputable suppliers.

2.2. Animals and treatments

Male Wistar rats at 4-6 weeks and weighing 180-220 gr were acquired and randomly assigned to six groups (Table 1). According to the instructions of the ethics committee for working with laboratory animals approved by Shiraz University of Medical Sciences (IR.SUMS.AEC.1401.110), mice were transferred to the animal care center of the Faculty of Pharmacy two weeks before the start of the experiment to familiarize themselves with the laboratory conditions. The conditions of keeping the mice included the animal room with a temperature of 22 to 25 °C and a humidity of 60% and light and dark periods of 12 hours alternately, with stress induction and AHR ligand including FICZ with 500 µg/kg (16) concentration and tryptophan with 50 mg/kg (17) concentration treatment conducted over one month. Fecal samples were collected for microbial analysis, and behavioral assessments were performed using forced swimming and splash tests.

Social defeat stress(SD) was induced using established protocols involving exposure to dominant male rats (18). Forced swimming test evaluated depression-like behavior by observing swimming, floating, and struggling, while splash test assessed grooming behavior changes (19).

Stools of animals from different groups were collected in a sterile tube during the experiment and immediately after collection, It was quickly frozen with liquid nitrogen and then stored in a freezer at -70 °C. Stool samples were cultured in MRS culture medium and biochemical and morphological tests were performed for microbial identification. DNA extraction (using a DNA extraction kit for gram-positive bacteria and according to the manufacturer's instructions) and PCR were used for further analysis, with agarose gel electrophoresis, which confirms the integrity of the DNA.

The sequence of primers used was, F: Lb1-F, 5′ -AGAGTTTGATCATGGCTCAG-3′ and R: ′Lb2-R, 5′ -CGGTATTAGCATCTGTTTCC3(20).

The temperature program of the thermocycler was set as follows: 5 min at 95 °C for one cycle, then 60 s at 95 °C, 30 s at 56.2 °C and 50 s at 72 °C, for 35 cycles, In the last step, the final extension was done at 72 °C for 5 minutes and one cycle.

2.3. Statistics

All studies were performed in at least two independent experiments. Data are expressed as mean ± SD. T-test was used to compare between two experimental groups. Probability values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Forced Swimming Test - First Session

Comparing the swimming behavior index

Table 1. Number of rats used in each group.

Groups	Control	SD	FICZ	FICZ+SD	TRP	TRP+SD
Number of rats	6	7	6	6	5	5

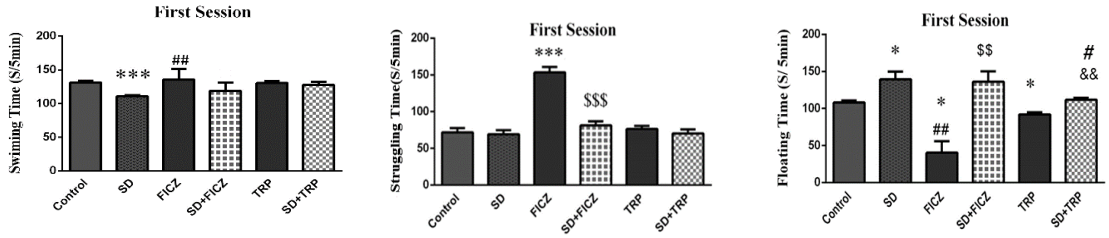

Figure 1. Forced swimming test on the first session. A forced swimming test was performed to evaluate depressive-like behavior. The duration of immobility was considered as an indicator that determines the level of despair and depression, and on the contrary, the swimming rate of mice was considered as a low level of depression. Data are expressed as mean±SD. (* vs, control; # vs, SD; $vs, FICZ; & vs, TRP) (* P<0.05, ** P<0.01, *** P<0.001).

on the first day between the control and SD groups revealed significantly reduced swimming in the SD group (p<0.001). Conversely, the FICZ group exhibited higher swimming indices compared to the SD group (p<0.01). Notably, buoyancy analysis showed higher levels in the SD group than the control (p<0.05),with the FICZ group exhibiting the lowest buoyancy. The TRP+SD group demonstrated increased immobility compared to TRP (p<0.05), while buoyancy was higher in SD compared to TRP+SD (p<0.05). Comparing FICZ+SD with FICZ, buoyancy was significantly higher in FICZ+SD (p<0.01). Struggling duration in the FICZ group was significantly higher than control (p<0.001), FICZ+SD showing lower struggling than FICZ (p<0.001) (Figure 1).

3.2. Forced Swimming Test - Second Session

Significant differences persisted on the second day, with lower swimming observed in the SD group compared to control (p<0.001). Comparing SD with FICZ+SD, swimming was lower in SD (p<0.05). Buoyancy analysis showed higher levels in control compared to FICZ (p<0.05) and lower in FICZ compared to SD (p<0.001). Comparing FICZ+SD with FICZ, buoyancy increased in FICZ+SD (p<0.001). Struggling duration was lower in SD than control and higher in FICZ than control (p<0.01). Comparing FICZ+SD with SD, struggling was significantly higher in FICZ+SD (p<0.01) and lower in FICZ+SD compared to FICZ (p<0.05) (Figure 2).

3.3. Splash Test

Self-grooming time was significantly lower in SD compared to control and FICZ (p<0.05) Conversely, FICZ+SD exhibited higher self-grooming compared to SD (p<0.05) (Figure 3).

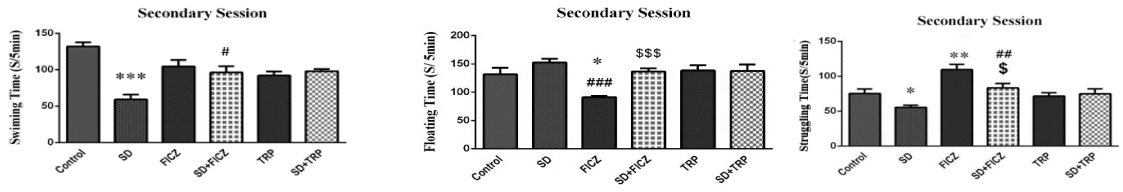

Figure 2. Forced swimming test on the second session. A forced swimming test was performed to evaluate depressive-like behavior. The duration of immobility was considered as an indicator that determines the level of despair and depression, and on the contrary, the swimming rate of mice was considered as a low level of depression. Data are expressed as mean±SD. (* vs, control; # vs, SD; $vs, FICZ; & vs, TRP) (* P<0.05, ** P<0.01, *** P<0.001)

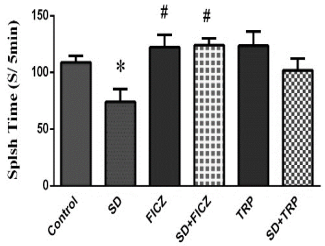

Figure 3. The time spent on the behavior index of cleaning and self-grooming in the splash test. Cleaning and self-grooming behavior were recorded as an indicator of caring and motivational behavior. Data are expressed as mean±SD. (* vs, control; # vs, SD) (* P<0.05, # P<0.05)

3.4. Biochemical and Morphological Tests

Morphological and biochemical tests confirmed the presence of Lactobacillus species. Oxidase and catalase tests were negative, and Gram staining revealed non-spore-forming, Gram-positive, rod-shaped bacteria (Figure 4, 5).

3.5. PCR and Electrophoresis

PCR products identified Lactobacillus species, consistent with the positive control Lactobacillus plantarum (190 bp band) (Figure 6).

4. Discussion

The study explores the impact of AHR ligands on the microbiota-gut-brain axis and its association with depression. FICZ, with its strong affinity for AHR (21), exhibits various physiological effects including hippocampal neurogenesis enhancement and memory improvement (22). Additionally, the brainstem and some hippocampal nuclei were shown to express much higher levels of AHR than other regions. FICZ is an ideal substrate for cytochromes CYP1A1/1A2 and 1B1 and therefore participates in the autoregulatory loop that maintains low steady-state concentrations. FICZ metabolism therefore fits the proposed autoregulatory model in which AHR signaling activated by various ligands is regulated by parallel induction of metabolic enzymes (3, 23). In the FST behavioral test of the present study, rats of the social defeat (SD) group showed the highest level of immobility and the lowest duration of swimming in both sessions of the test. In addition, the FICZ group showed the highest amount of active behaviors including swimming and fighting.

Figure 4. Colonies of bacteria on MRS agar after 72h incubation at 37ºC.

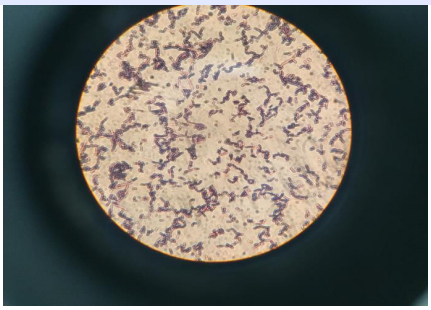

Figure 5. Gram-positive bacteria were examined by light microscope with 100x magnification power.

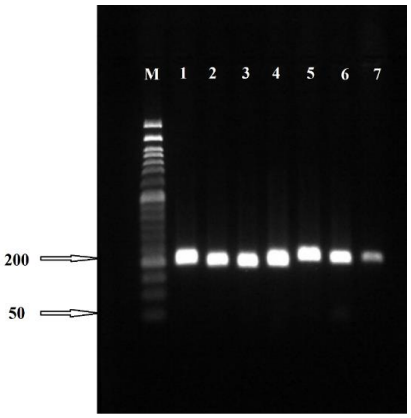

Figure 6. The gel electrophoresis of PCR product. M: 50 bp DNA ladder, line1: positive control, line 2: TRP, line 3: TRP+SD, line 4: FICZ, line5: FICZ+SD, line6: control, line 7: SD

A study by Mohammadi *et al.* is in agreement with our study and shows that AHR activation by FICZ improves hippocampal-dependent memory and learning tasks (4). In the behavioral splash test, the FICZ and FICZ+SD groups showed the longest cleaning time and self-grooming behavior, and the SD group showed the lowest amount of self-grooming behavior. Vidal *et al.*'s research shows that social defeat stress during adolescence (45-58 days after birth) can lead to social avoidance behavior even 7 weeks later. These studies show that repeated exposure to social defeat stress consistently results in prolonged social avoidance behavior that serves as an index to measure social anxiety in animals (24). A balanced AHR activity is crucial for hippocampus-dependent contextual memory (25). Tryptophan deficiency is linked to depression (26), with its metabolism through the kynurenine pathway playing a role in various diseases, including neuropsychiatric disorders like depression and schizophrenia (27). Stress-induced alterations in the kynurenine pathway can lead to depression-like behaviors, metabolites of the KYN pathway have neurotoxic/neuroprotective activity. 3-Hydroxy kynurenine and quinolinic acid have neurodegenerative effects, while kynurenic acid has neuroprotective effects (28-31). The balance of kynurenic acid and quinolinic acid metabolism in the brain is associated with certain regions, especially the hippocampus (30). A group of researchers found that a lower “neuroprotective index,” or kynurenic acid/quinolinic acid ratio was associated with reduced hippocampal volume in patients with major depression (30). The microbiota-gut-brain axis plays a crucial role in depression (32), with stress disrupting the microbiome and increasing susceptibility to depression (33). Clinical studies reveal differences in the gut microbiota of depressed patients, with decreased microbiota diversity and abundance (32). Lactobacillus species, including Lactobacillus plantarum, have been shown to reduce stress and anxiety (34, 35). The beneficial effects of Lactobacillus species on mood may rely on several mechanisms, including modulation of kynurenine production, the hypothalamic-pituitary-adrenal (HPA) axis, and the immune system Stress exposure can affect microbial populations, including lactobacilli that are closely associated with the colonic mucosa potentially impacting mental health (35). Galley, J. D et al.'s study showed that less than 2 hours of exposure to stressors is sufficient to significantly alter the composition of the microbiota associated with the colonic mucosa. Also, 2 hours of exposure to stressful factors decreased the relative abundance of the Lactobacillus species (36). The results of the present study are consistent with this study. Our study, according to the PCR results, showed a relative reduction of Lactobacillus species in the SD group. Therapies targeting the gut microbiota and the microbiota-gut-brain axis hold promise for depression treatment and prevention. In conclusion, FICZ and TRP show potential in alleviating depression induced by social failure, while exposure to stressors can reduce the frequency of Lactobacillus spp. The study underscores the importance of microbiota-based therapies in depression management.

More studies are needed to determine whether the stress caused by social defeat affects the population of Lactobacilli in other groups, as well as the population of other bacterial species.

From a behavioral and cognitive point of view, improving the quality of conducting existing tests and how to extract and record data from them can increase the reliability of the results.

In behavioral studies, many factors affect the study, some of which are unknown to us, such as season, age, etc.

5. Conclusion

FICZ and TRP show promise in alleviating social defeat-induced depression. In the examination of the TRP group in the FST test, after the FICZ group, the TRP group had the lowest level of buoyancy among the groups, which seems to be the lower effect of TRP than FICZ on depression caused by social defeat due to the possible entry of TRP into the kynurenine pathway. Stress exposure reduces Lactobacillus spp. frequency, underscoring the potential of microbiota-based therapies in depression management.

Compliance with Ethical Standards

Compliance with ethical standards was ensured throughout the study, following the guidelines of the Ethics Committee of Shiraz University of Medical Sciences. (Ethics code: IR.SUMS.AEC.1401.110)

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Conflict of Interest

The authors declare no conflict of interest.

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