Original Article

Restoration of Morphine-Induced Amnesia by *Pediococcus acidilactici*, an Indigenous Probiotics of Iran, in Male Wistar Rats

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Abstract

Background: Probiotics are living microorganisms that have beneficial effects on the microbial balance of the host intestine (human and animal). Studies demonstrated that intestinal microbiota maybe affect the hormones of brain and nervous through the vagus nerve. The goal of this study was to investigate the impact of Lactobacillus *Pediococcus acidilactici*, indigenous probiotics of Iran isolated from traditional dairy products, on passive avoidance learning of male Wistar rats. **Materials and Methods:** In this research, 80 male Wistar rats weighing from 80 to 100 grams were used. For induction of amnesia, morphine was used as an intraperitoneal injection. Afterward, the mice were with 0.1 ml of milk alone or containing 109 CFU/ml of. *Pediococcus acidilactici* for 8 months. In this study, a non-active avoidance learning behavioral test was used to test long-term memory in Wistar rats. **Results:** Results displayed that in the control group, morphine (1 mg/rat) significantly decreased learning. In the groups receiving probiotic and probiotic extract plus morphine, there was a significant difference in learning circumstances with the control group. **Conclusion:** The results of this study depict the beneficial effect of *Pediococcus acidilactici*, a native Iranian probiotic isolated from dairy products, in the learning quality of non-active conditional avoidance in rats induced by morphine. This indicates the effect of oral probiotics on improving memory and learning.
Keywords: Amnesia, Probiotic, Morphine, Lactobacillus *Pediococcus acidilactici*, Wistar rats.

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Introduction

The issue of memory and learning is important when the disease of amnesia and mental impairments affect one in every five people over the age of 65 with aging (1). Such diseases are progressive brain disorders that are rapidly destroying diagnosis and memory skills. These kinds of diseases, due to its specific characteristics, have bulky economic and emotional pressure on patients and families as well as governments (2).

Accordingly, it can be said that the formation and recall of memory are mediated by several neuronal pathways, which are controlled by various neurotransmitters like morphine. Several factors have shown that morphine has a damaging effect on memory and learning flows in laboratory animals (3, 4).

Studies were done by Iscavrida et al. in 1979 and Castellano et al. in 1993 and Diaz et al. in 1985 demonstrated that opioid agents, including morphine, have an inhibitory effect on memory formation and avoidance learning in laboratory animals (5, 6). Diaz et al. concluded that such disorders by opioid agents are dose-dependent (7). In a study of the effect of the interaction of morphine plus nicotine on memory in nicotine-susceptible mice, it was demonstrated that morphine would suppress memory (8). On the other hand, evidence has a display that there may be an

association between opioids and some types of learning and memory processes (9).

The hippocampus "a part of the limbic system", which is responsible for memory and formation of the brain is ahead of our past experiments (10). It protects the past memories in the short or long term. Many studies have exhibited that morphine exerts its effects directly or indirectly through various neurotransmitters such as acetylcholine, dopamine, opioid peptides, and amino acid mediators (11). Frequent administration of morphine decreased the speed of learning in the water maze and impairs learning (Figure 1) (12).

Probiotics are dietary and pharmaceutical supplements made from living microorganisms exert beneficial effects on the host, and improve the food conversion rate and weight gain (13, 14). Lactobacillus and Bifidobacterium are commonly used as probiotics. It should be noted that different bacterial strains have also different efficacies (15).

Most studies used Bifidobacterium Sp. and Lactobacillus Sp. with doses between 109 and 1010 colony-forming units for 2 weeks in animals and 4 weeks in humans. These probiotics revealed efficacy in improving anxiety, depression, autism spectrum disorder (ASD), obsessive-compulsive disorder, and memory abilities(16).

In this study, we intended to investigate the effect of *Pediococcus acidilactici*, indigenous probiotics of Iran isolated from traditional dairy products, on passive avoidance learning of male Wistar rats.

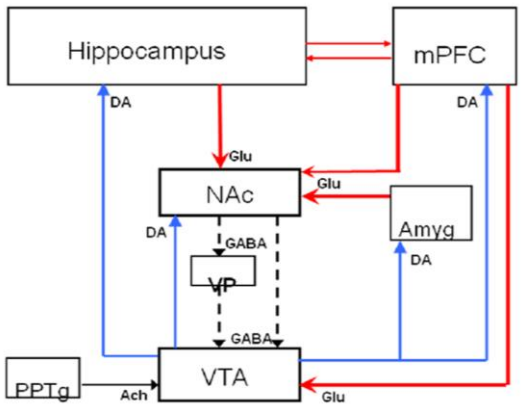

Figure 1. Schematic diagram of the multi-core neuronal connecting pathways of the limbic system involved in inhibitory avoidance memory.

Methods

Mice. In this study, we used 80 male Wistar rats (weighing 80 - 100 g), purchased from the Pasteur Institute of Iran. Mice were kept in of the Department of Physiology's animal room, under standard temperatures (22±3 °C) and 12 hr light/12 hr dark cycle for 32 weeks. Eight rats were placed inside each shelf and food and water were given to the rats and every 3 days the shelves were cleaned.

Before test initiation, rats were allowed for one week to adapt themselves to the animal room conditions. Animals were treated every day in order to avoid stress. Each animal was used only once and placed in 8 mice per each group (10 groups). All experiments were performed for two consecutive days in one day of training and one day of testing. Maximum care was taken in order to keep the condition similar for all rats.

The inactive avoidance mechanism, model or shuttle avoidance device consisting of a two-part box divided into two identical walls is embedded inside the wall between two parts of a sliding door of dimensions 7×9 cm.It can be opened and closed if it is necessary. This device has two parts, one white and the other black, the white part is made of non-transparent glossy plastic and illuminated by indirect sunlight fluorescence. The dark part of the device is in the floor with 1cm steel bars, which are attached by the wire to the stimulating device (shock generator), thus allowing the electrical shock transfer operation to be made to the desired animal. All of these experiments were carried out in a fairly dark and noiseless room.

Behavioral tests. An inhibitory avoidance method was used to study memory in Wistar rats for two consecutive days. On the first day, the training day included teaching animals to the shuttle box and examining the amount of memory of the trained animals on the second or the following day.

Training phase. In the avoidance behaviors model, at 8:00 Am, the animals were taken to the workroom for half an hour in the morning, and we were waiting to get the animals to be accustomed to the conditions of the room. Then each animal was placed gently on the bright side of the Shuttle Box system and allowed them to become familiar with this circumstance for 5 seconds. Then, by opening the

sliding door that was embedded in the middle of the device between its bright and dark part, we allowed the animals to enter the dark part of the device. Immediately after entering the animal into the dark part, when the hind legs and the tail of the animal were entered the dark part, the sliding door was closed and the animal was pulled out of the machine and returned to its original cage.

We eliminated mice that were delayed more than 120 seconds at the entrance to the dark part from further evaluations. Mice that were approved and entered the dark part of the device for less than 120 seconds, were immediately subjected to the injection of morphine. After 30 minutes, the animal was transferred to the white section of the shuttle box, and after the 5 seconds, a sliding door was opened to allow the animal to enter the dark part of the device. With the animal entering to the dark part, the sliding door behind the animal was closed and the animal was stimulated with an intensity of 1 milliampere for 3 seconds, which was transmitted by the stimulator to the steel bars located at the bottom of the dark part of the shuttle box. Ten seconds after receiving an animal shock, the animal was withdrawn from the machine and was returned to its primary cage. After 2 min, training was performed on rats undertaken shock. At this stage, the mouse was transferred to the white part for the first time and after 5 seconds the sliding door was opened and the animal was allowed to go into the dark part of the machine. The delay of 120 seconds in entering the dark part of the device was considered as a successful learning for the animal. In case of a delay of less than 120 seconds in entering the dark part, after entering the dark section, the sliding door was closed and the animal received a shock for the second time. After taking the animal out from the device and passing 2 mins, the animal's memory was checked again. When there was successful learning and no animal entry into the dark part of the machine in the less than 120 seconds, the animal was taken out from the machine and returned to the primary cage. But if after this stage and after the second shock, the animal was entered to the dark part in less than120 seconds, the rat was eliminated from further evaluations. Maximum training for each rat was considered three times.

Test step or memory evaluation. During the test session, which is carried out 24 hours after the training, we did not have any electrical stimulation or drug injection at this session (test session). To examine the memory, we put each rat on the device's light section and after the 5 seconds of being an acquaintance, the shuttle box was opened and rats were allowed 300 seconds to enter the dark part of the device. The animal's delay in entering into the dark part of the device was considered as a criterion for measuring the memory rate. The full memory as then highest amount of latency to enter the dark section was considered to be 300 seconds.

Probiotic preparation. In this research, the Iranian indigenous *Pediococcus acidilactici* isolated from dairy products, which was documented by Ebrahimi et al. (17) was used for gavage after biochemical characterization and genetic determination with the interception code. The bacteria were first cultured on MRS medium (Lactobacilli MRS Agar) and incubated at 37 °C for 24 h. At the end of the growth period, the optical absorption of the medium was determined at 600 nm wavelength and the dilution series from bacterial culture was prepared. Surface treatment method was used to determine the number of living bacteria in the culture media. The purpose of this section was to provide a definite and stable number of viable bacteria throughout the entire test period.

During the 32-week period of the experiment, fresh bacterial dilution was prepared every day. The rats receiving bacteria as well as the supernatant, daily were gavaged with this suspension of bacteria (bacteria + a certain volume of milk), while the control group received the same amount of milk.

Drugs. All drugs were prepared on a test day and freshly prepared. Morphine was purchased from Daroopakhsh Corporation. Morphine was dissolved in saline; an intravenous injection (IP) was used to investigate the effects. First, the animal was weighed and the desired doses for each 100 gr were intraperitoneally injected. About 1 cc of morphine was injected half an hour before rates were instructed.

The bacteria and also the metabolite of the bacterium were gavaged to the mice for complete testing. In addition, fresh milk, as a diluent for *Pediococcus acidilactici*, was prepared daily.

Examined parameters. In order to increase the

accuracy of the effect of *Pediococcus acidilactici* on memory in Wistar male rats, the following factors were measured.

- Time of avoidance in entering to the dark section of the ShuttleBox

- Time of stay in the dark room

First experiment: Pre-test morphine injection on inhibitory avoidance memory in Wistar rats.

In this experiment, four animal groups were used to study the effect of morphine on memory. Animals were received milk for one month through gavaging. Intraperitoneal administration of different doses of morphine (0, 1.25, 2.5, and 5 mg/kg) was conducted 30 mins before instructions.

Second experiment: Effect of *Pediococcus acidilactici* on the delay in entering to the dark room on the test day

Third experiment: The effect of *Pediococcus acidilactici* on the time spent in the darkroom on the day of the experiment.

Statistical methods. In these experiments, one-way ANOVA and Tukey tests were used to measure the significant difference between the groups through SPSS v.25. The data were represented as a mean ± standard deviation for each group. In all stages, P <0.05 was considered a significant level.

Results

First experiment: The results of pre-morphine injection on inhibitory avoidance memory in Wistar rats. The results showed that pre-injection of morphine (5 mg/Kg) alone leads to deterioration of inhibitory avoidance memory (P <0.001; Figure 2).

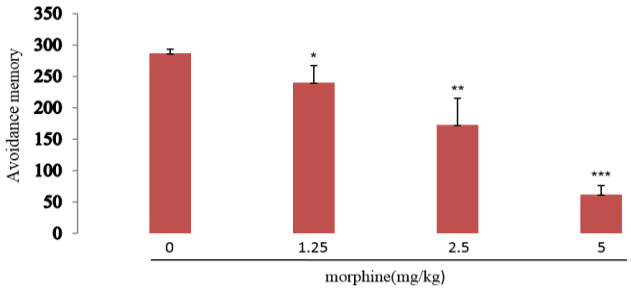

Figure 2. The effects of pre-training morphine administration on inhibitory avoidance memory. The data is presented as Median ± interquartile range (IQR). ***P <0.001 in comparison with the saline group..

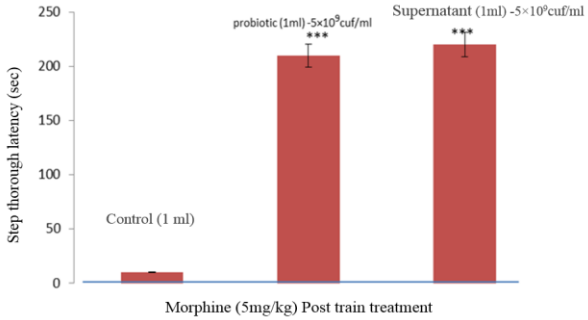

Figure 2. Comparison of the initial delay in entering to the dark room between the group receiving probiotics and morphine and the group receiving the metabolite of bacteria (0.1with a concentration of 109 CFU/ml) with morphine and the group receiving milk plus morphine. The data is presented as Median ± interquartile range (IQR). ***P <0.001 in comparison with the saline group.

Second experiment: Effect of Pediococcus acidilactici on the delay in entering to the dark room on the test day. The results demonstrated that there is a significant initial latency to enter the dark room between the group receiving bacteria plus morphine (5 mg/kg) and the group receiving the metabolite of bacterial plus morphine compared to the group receiving the milk in combination plus morphine. The one-way ANOVA demonstrated a significant increase in latency for entering the darkroom (P<0.001; Figure 3).

Third experiment: The effect and the comparison between the group receiving probiotics and morphine and the group receiving the metabolite of bacteria (0.1 ml plus a concentration of 109 CFU/ml with morphine with the group receiving the milk plus morphine on the time spent in the darkroom on the test day. The results demonstrated

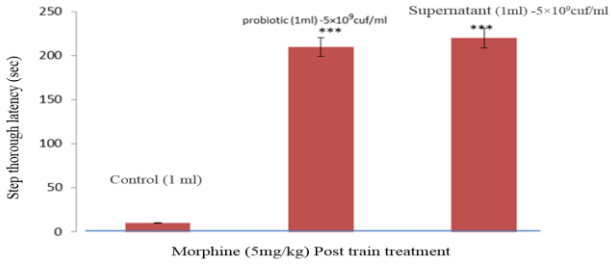

Figure 4. Comparison of the time remaining in the dark room between the group receiving the bacterium plus morphine (5 mg/kg) and the group receiving the extract of the bacterium with morphine compared with the group receiving milk plus morphine showed a significant difference according to one-way ANOVA. The data is presented as Median ± interquartile range (IQR). ***P <0.001 in comparison with the saline group

that the group receiving the bacterium and morphine (second column) and the group receiving the *Pediococcus acidilactici* plus morphine (third column) showed a significant decrease in the time spent in the darkroom compared with the group receiving milk plus morphine (P<0.001; Figure 4).

Discussion

Structures such as hippocampus are involved in avoidance inhibitory as a long-term memory,. Hippocampus is one of the main areas involved in learning and memory (18). As noted in previous studies, the hippocampus is rich in corticosteroid and opioid receptors that are involved in memory (19). The hippocampus is part of the prefrontal cortex in the mammalian brain and is the main structure in spatial learning and memory consolidation. The hippocampus has long been known as a memory and learning activity structure (20).

One of the known actions of opioids (morphine) is the destruction of memory. CB1 receptors are located at the axonal terminus of the pre-synaptic neurons, and the activation of these receptors reduces the activity of mediators (21). The effect of CB1 on opioids can reduce the release of GABA, glutamate, dopamine, acetylcholine, norepinephrine in the hippocampus (22). It can be concluded that the lack of these mediators can lead to memory loss.

GABA is the most important inhibitory neurotransmitter that is released by bringing neurons and inhibition interfaces on the cell body and dendritic cells of pyramidal neurons, and its effect depends on the type of post-synaptic receptor (23). In addition to GABA, there are other inhibitory neurotransmitters, including somatostatin, serotonin, and histamine in the hippocampus (24). It has been reported that the amount of GABA in mice changes with probiotic intake (25). Both excessive decrease and increase of GABA in the brain lead to depression and anxiety, respectively (26). In 2005, the performance of the loop between the hippocampus and the VTA was determined, which was involved in long-term memory regulation (27). The VPA has a dopaminergic neuron that reaches from the VTA to the hippocampus, and the downstream path is composed of the accumbens nucleus and the lateral pallidum. Then, from the accumbens nucleus and the VP, the outputs of the GABAergic that are returned to the VTA are able to have inhibitory control over their target positions. The accumbens nucleus also receives dopaminergic inputs from VTA. It also receives many glutamatergic inputs from the medial prefrontal cortex (mPFC), thalamus, and amygdala hippocampus (28).

According to the previous reports and the results obtained in the recent study, it can be concluded that morphine leads to inhibition of glutamatergic system and therefore, due to the removal of the inhibitory effect of glutamate on the GABAergic system, this system is activated and leads to the induction of forgetfulness (29).

However, probiotics can activate the serotonergic system and inhibit the GABA system. It can be concluded that probiotics stimulate the serotonergic system by inducing memory. By altering the gene expression of GABA receptors, they can directly inhibit the GABA system and lead to more than expected memory retention(30).

Serotonin in mammals has a complex role in memory in various behavioral models. Of course, there are some incongruous results that make the comparison difficult. However, serotonin plays a role in memory and learning by its receptors (31). Different results may be related to the type of receptor, the place where the process (systemic or central), the drug, the medication time, and the behavioral tests performed. Although there are a limited number of serotonergic agonists and serotonergic receptor antagonists, evidence suggests that serotonin is a synaptic plasticity linker in pre-synaptic and post-synaptic (such as the message transmission pathway) during memory and learning formation. Researches have shown that serotonin moderates post-synaptic memory. Probiotics also affect GABA through the production of serotonin, which results in the loss of GABA activity, which in turn reduces stress and induction of memory. In this way, any factor that suppresses GABA will enhance memory and learning and, as we know, morphine produces a stimulant effect on GABA and somehow induces forgetfulness. On the contrary, when the serotonergic system is activated and serotonin is secreted, it inhibits GABA and boosts memory. Morphine consumption may delay the development of the hippocampus in the rat's embryo,

which is observed in increasing of the cell number in this area to different layers and decreasing the diameter of each layer (32).

Morphine can also damage brain cell communication in learning and memory areas. Morphine reduces neuronal involvement in the hippocampal region and reduces short-term memory (33). Proven long-term use or high doses of morphine also affects cells in learning areas and memory in the brain. Other studies have shown that learning and memory in laboratory animals are affected by their opioid antagonists, and it has been shown that the mean values of morphine (5-10 mg/kg), when it administered prior to the training, has a damaging effect on memory during the passive avoidance method (34).

Studies on the beneficial effects of probiotics on mental health and the hypothalamic-pituitary-adrenal axis in petrochemical workers demonstrated that consumption of probiotic yogurt or a multispecies probiotic capsule had a beneficial impact on mental health parameters in petrochemical workers (35).

Recent research has shown that probiotics can modify morphine effects and improve the negative effects of morphine on memory. Laboratory studies have shown that the use of probiotics can improve mental states and reduce anxiety in patients (36). In other words, probiotics can affect emotional state and probiotic therapy can reduce the measurable negative effects of morphine. The beneficial effects of probiotics in preventing stress-induced memory loss is exerted through a modification of the hypothalamus-pituitary-adrenal gland (hypothalamus-pituitary-adrenal). Since studies on cognitive effects of probiotic are rare, further studies are needed to explore this finding in other models of learning and memory measurement as well as in other strains (37).

The overall results of this study indicated that Lactobacillus spp. possesses characteristics that are prone to probiotic use and extensive use for improving human and medical problems and higher clinical trials. Morphine can damage memory, depending on the amount and duration of use. Morphine has a profound effect on learning and memory and prevents complete learning.

In human studies, there is evidence of the role of intestinal microflora in the functioning of the central nervous system (CNS), which indicates that oral consumption of probiotics and the balance of the gastrointestinal microbial population have beneficial effects on mood and mental distress and memory (38). The results of this study showed that the use of this probiotic (*Pediococcus acidilactici*) as well as its metabolites improved and prevented the destruction of the learning and memory process in Wistar rats using morphine. As well, it could decrease the effects of harmful chemicals (morphine) and physiologic agents that could have a harmful effect on the brain and especially the hippocampus. This means that mice received probiotics plus morphine had no delay in finding the door to enter the dark room of the shuttle in comparison to the control mice received no probiotics but received morphine. However, the group who received neither probiotics nor probiotic metabolites but received morphine, entered the dark room with delay and needed more time.

Conclusion

Therefore, it can be concluded that probiotics and their metabolites can influence on the learning process of animals in the control group and improve the performance of the mice upon entering the dark room. In addition, probiotics were effective in maintaining the memory of Wistar rats, and the probiotic group showed better performance than the negative control group. The *Pediococcus acidilactici*, identified by 16srRNA sequencing method, has probiotic properties as well as the beneficial effects in memory induction and learning in Wistar rats and can be very useful in this regard.

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Conflicts of Interest

The authors have no conflict of interest to declare.

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