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Research paper

Protective role of Eugenol against the destructive effects of lead on conditioned fear memory in male rats with post-traumatic stress disorder-related behavioral traits

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ABSTRACT

Introduction: Post-traumatic stress disorder (PTSD) is a consequence of living in today's stressful society. Patients have difficulty forgetting traumatic events. lead pollution has many effects on the nervous system, one of which is memory and learning disorders. The herbal medicine Eugenol has a beneficial effect on memory.

Aim: This study aims to investigate the protective effect of Eugenol on lead-induced memory impairments in stressed rats.

Methods: In the first experiment, the animals were divided into three groups: SPS+Saline, SPS+Pb, and naïve. The SPS+Saline, SPS+Pb groups received normal saline and lead through gavage for 21 days, while the sham group remained untreated. Rats were subjected to the modified single prolonged stress model. Memory tests were conducted one week later, evaluating freezing levels in three consecutive tests over three days. In the second experiment, rats were divided into a SPS+Pb+Saline and three treatment groups. The SPS+Pb+Saline group received daily saline injections, while the other groups received different doses of Eugenol (25, 50, and 100 mg/kg). Memory tests similar to the first experiment were conducted.

Results: The results showed significantly higher immobility levels in the SPS+Saline and SPS+Pb groups compared to the sham. Additionally, the SPS+Pb group had a significant higher immobility compared to the SPS+Saline group. In the second experiment, the SPS+Pb+EU 25 group showed a significant lower freezing compared to the SPS+Pb+Saline group. Additionally, freezing in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups was significantly higher than in the SPS+Pb+EU 25 group. The SPS+Pb+EU 50 group showed a significant higher freezing compared to the SPS+Pb+Saline group.

Conclusion: lead acetate exacerbated memory impairments in stressed rats and Eugenol, particularly at a dose of 25 mg/kg, improved these impairments. Therefore, Eugenol has the potential to partially reduce the negative effects of lead on memory in individuals with PTSD.

Introduction

Lead is a shiny, bluish metal, very soft, flexible and a relatively weak conductor, it has excellent flexibility and high resistance to corrosion (Andrade et al., 2015). Therefore, it is widely used in industry, so that it is the most widely used metal in the world after iron. The wide application of lead, leads to its considerable dispersion in air, water, food and soil. Lead particles enter the human body and animals through skin, inhalation and ingestion and accumulate in different tissues. High concentrations of lead cause many toxic effects in different body systems. Abdominal pains, kidney damage, anemia, encephalopathy and mental retardation in children are among the complications of lead poisoning (Karrari et al., 2012; Dignam et al., 2019). One of the most important effects of lead poisoning on the function of the nervous system is its effects on the reduction of nerve message transmission, the destruction of the myelin sheath and Schwann cells. Lead also has a destructive effect on learning and memory. One of its mechanisms is the destruction of N-methyl-D-aspartate (NMDA) glutamate receptors.

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NMDA receptor plays an important role for controlling synaptic plasticity and mediating learning and memory functions in memory and learning (Sanders et al., 2009).

Stress is a normal physical response to challenging or new situations that can be caused by various life experiences. Stress can cause a variety of physical health problems, including depression or anxiety, heart disease, irritable bowel syndrome, sleep problems, and more (Health AIo, 2022) In today's life, people are subject to a greater number and variety of stressful situations that can be the cause of serious psychological damage. Any serious life-threatening event or stress, such as natural disasters, war, accidents, rape, epidemics, etc., can be traumatic (Richter-Levin and Sandi, 2021). Traumatic injury, with its physical, emotional, cognitive and financial consequences, can affect people's lives for a long time. Not everyone reacts to trauma in the same way. One of the most common and well-known mental health conditions associated with trauma is post-traumatic stress disorder (PTSD). PTSD is a chronic condition in which fear, anxiety, and memories of a traumatic event remain with a person for a long time and interfere with daily life (Health AIo, 2022). Traumatic events cause disease symptoms by affecting multiple endocrine (such as the hypothalamus-pituitary-adrenal axis), neurochemical (such as catecholamines) and brain circuits (Sherin and Nemeroff, 2011). PTSD is a disorder in which the memory of the traumatic event dominates the patient's thoughts and reduces their enjoyment of life. Memory disorders in PTSD are one of the prominent features of this disease and one of its diagnostic symptoms. Clinical studies of patients with PTSD revealed that these patients suffer from two important disorders related to memory: first, re-experiencing and constantly recalling traumatic events and second, avoiding the stimuli that caused the trauma, even though they know that the traumatic event will not be repeated. These people do not have the ability to turn off the memory of the trauma. In PTSD, trauma leads to a reduction in the volume of sensitive brain areas such as the hippocampus and prefrontal cortex through oxidative stress and apoptosis. The normal neural connections between the hippocampus, amygdala and prefrontal cortex are altered and thus memory dysfunction is created (van der Kolk, 2000). The use of neuroprotectants may be one of the ways to prevent nervous system disorders such as PTSD. Although many industrial drugs are used for this purpose today, the use of compounds of natural and herbal origin is usually the least complicated method of prevention.

A phenylpropanoid compound of interest is eugenol, specifically known as 4-allyl-2-methoxyphenol (SPS+SALINE0H12O2). Eugenol exhibits weak acidic properties and demonstrates limited solubility in water, while being soluble in organic solvents. At room temperature, it presents as a pale yellow, viscous, oily liquid with a distinct clove aroma and a spicy taste. This compound finds widespread application in the flavoring industry, as well as within the pharmaceutical, agricultural, aromatic, and cosmetic sectors (Mohammadi Nejad et al., 2017).

Eugenol is naturally present in various aromatic plants such as nutmeg, Saigon cinnamon, and sweet basil (ElGhannam et al., 2023). However, its primary natural source is Eugenia caryophyllata. Notably, eugenol possesses remarkable pharmacological properties, including anti-inflammatory, antiviral, anti-flatulent, anti-fungal, anti-tumor, anti-seizure, and pain-relieving effects (Nisar et al., 2021). Furthermore, it exhibits memory-enhancing attributes and displays potent antioxidant activity (Barboza et al., 2018).

Considering its potential as a neuroprotective substance, our research focuses on investigating the impact of eugenol on memory impairment in stressed rats exposed to lead poisoning. By exploring the effects of eugenol in this specific context, we aim to shed light on its ability to protect and support cognitive functions

Materials and methods

Animals

In the present research, 70 male Wistar rats with an approximate weight of 180–200 g were used. Animals were kept in five cages of 4–5 at a temperature of 20–22 degrees Celsius with 12 hours of darkness and 12 hours of light and with free access to food and water in this environment. The animals were obtained from the breeding colony of Iran's Pasture Institute. At least one week before starting any lab work, give them time to acclimate to their surroundings. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were carried out under the supervision of the ethics committee of Damghan University and were approved by the research ethical standards for the care and use of animals in Damghan University."

Contextual fear conditioning apparatus

The rodent fear conditioning system from Noldus, the Netherlands, was used to study contextual fear conditions. This system consists of a box whose walls and ceiling are made of transparent Plexiglas. The bottom of the box consists of 25 stainless steel rods (diameter 6 mm, spaced 12 mm apart) through which the shock can be delivered to the animal. The box is placed in a semi-acoustic enclosure. The chamber is illuminated by a lamp. Before and after each test, the interior of the chamber was cleaned with 5% ethanol to remove signs of previous contamination. Ventilation fans provided continuous background noise (68 dB) throughout the experiment. An EthoVision software program was used to collect, display and store all experimental data for "off-line" analysis.

Experiment materials and preparation method

Lead acetate (Sigma-Aldrich) was dissolved in distilled water at concentration of 20 mg/kg body weight of 2% solution and administrated to rats by gavage tube (Soleimani et al., 2017; Bazrgar et al., 2015; Elgawish and Abdelrazek, 2014).

Eugenol is a clear, injectable oily liquid available from Sigma. Due to its low polarity, eugenol is relatively soluble in water and organic solvents (Shang et al., 2021). Therefore, eugenol has been prepared by dilution in 0.9% normal saline for 15 minutes in the presence of gentle and indirect heat.

Based on preliminary research conducted at Damghan University's laboratory, three doses of eugenol were used in the study: 25 mg/kg, 50 mg/kg, and 100 mg/kg. These doses were administered intraperitoneally to the subjects.

PTSD animal model induction method

Induction of the modified SPS model (Wang et al., 2008):

Step 1: Animals were immobilized in a restrainer for two hours.

Step 2: After being released from restraint device, the animals are placed in a cylindrical container for forced swimming for 20 minutes.

Step 3: Fifteen minutes after the swimming session, the animals were anesthetized with diethyl ether.

Half an hour after the rats recovered from anesthesia, they were placed in in a conditioned fear system and received one electric shock of 0.5 mA for one second (Borghans and Homberg, 2015).

Evaluation method for conditioned fear memory

The evaluation method involves assessing the animals' response to a conditioned fear device. Many findings show that behavioral and cellular changes caused by SPS are time-dependent, and most changes are observed 7 days after exposure to the SPS method (Liberzon et al.,

1999); therefore, behavioral tests were performed one week after the animals experienced the modified SPS model. For the conditioned fear study, one week after the modified SPS, the animals were again exposed to the shock chamber for 5 min without further shock application and then returned to their home cages (test one). 24 h after the first test, the animals were again placed in the shock chamber for 5 min without shock to recall the conditioned fear memory (second test). The third test was similar to the second test and was administered twenty-four hours later. In other words, three fear memory tests were administered on three consecutive days, days 21, 22, and 23 of the first test and days 28–30 of the second test.

During the tests, the animals' behavior inside the conditioned fear device is recorded using EthoVision-3 software. The software is connected to the device to monitor and analyze the animals' behaviors, calculates the immobility (freezing) time exhibited by the animals. The immobility time is measured as a percentage of the total time spent in the conditioned fear device.

Open field test

Lead can affect an animal's activity level and lethargy. Therefore, we used the open field test to evaluate the animal's locomotor activity. The apparatus for this test consists of a cubic chamber (40×40×30 cm), the bottom of which is divided into 9 equal squares (3×3 cm). The walls of the box are made of Plexiglas. The box was placed in a room with indirect lighting. Two hours after the last memory test, the rat was placed in the box for 5 minutes and the number of times it crossed the lines between the squares was counted.

Experiment groups

First experiment groups

In this experiment, 30 rats were randomly divided into the following 3 groups:

Sham Group: The rat in this group did not undergo any experimental manipulation (no fear conditioning) or treatment. Memory tests were taken only from the animals of this group. They served as a baseline comparison for the other groups.

SPS+Saline: For three weeks beginning on the day 1 of the experiment, the animals in this group were orally gavaged daily with 0.2 cc of saline. The animals were subjected to a modified single prolonged stress model on day 14 of the experiment. Three memory tests were performed on them over three consecutive days one week after disease induction, that is, on days 21, 22 and 23 of the experiment. The duration of each test was 5 min.

Treatment group with 0.2% lead)20 mg/kg (SPS+Pb group: The animals in this group were subjected to the same experimental procedures as the SPS+Saline group, with the exception of receiving 0.2% lead acetate solution by gavage every day for three weeks (Fig. 1).

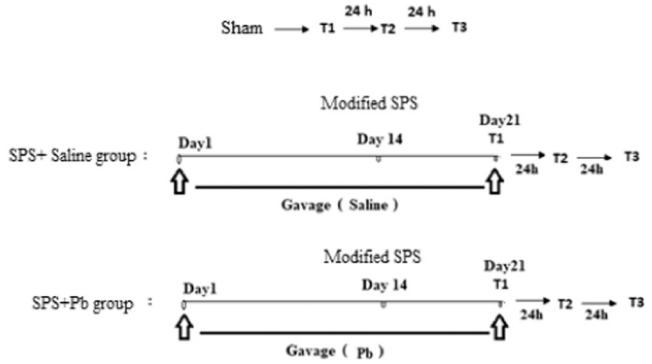

Fig. 1. The experiment steps in the first experiment groups.

The second experiment

The first experiment aimed to investigate the impact of chronic administration of lead acetate on the conditioned fear memory in male stressed rats. On the other hand, the second experiment focused on examining the influence of eugenol on the conditioned fear memory in rats previously exposed to lead acetate. To achieve these objectives, a total of 40 rats were randomly allocated into four distinct groups.

The SPS+Pb+Saline group in this experiment consisted of animals that received 0.2 cc saline (eugenol solvent) intraperitoneally once a day for four weeks, four days a week, starting from day 1. Additionally, starting on the seventh day of the experiment, they were given a daily gavage of a 0.2% lead acetate solution. These animals were then subjected to the modified single prolonged stress model, which is a method used to induce post-traumatic stress disorder (PTSD)-like symptoms in animals. On the 21st day of the experiment, the animals in this group were conditioned by receiving electric shocks in the FCS (fear conditioning system) machine. A week later, on the 28th day of the experiment, the animals were given three memory tests on three consecutive days. These memory tests were likely designed to assess their ability to recall and remember specific information or experiences related to the experiment.

The treatment groups were divided based on the dosage of eugenol given. The groups were labeled SPS+Pb+EU 25, SPS+Pb+EU 50 and SPS+Pb+EU 100, representing the eugenol doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg, respectively. The animals in these groups received eugenol injections at their respective doses for four weeks. The injections were administered four days a week, once a day (Fig. 2).

Statistical analysis

The data is presented as mean ± SEM. Behavioral data were analyzed with two-way analysis of variance. SPSS version 21 was used to analyze the statistical data. The general linear model for repeated measures was used for group comparisons of overall differences between groups. Tukey's post hoc test was performed to determine the source of detected significant differences. Values of $P < 0.05$ were considered significant.

Results

The results of the first experiment

The first experiment aimed to investigate the impact of lead exposure on the process of fear memory in male stressed rats. Over the course of three consecutive days, the freezing rate of animals within three distinct experimental groups—Sham, SPS+Saline, and SPS+Pb —was assessed

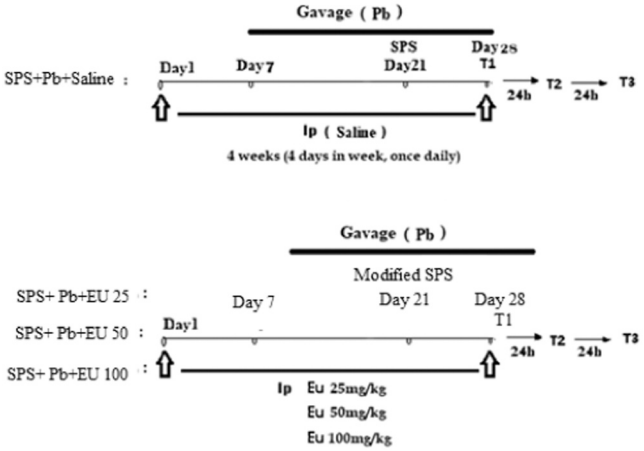

Fig. 2. The experiment steps in the second experiment groups.

during three memory tests. The SPS+Saline group was administered normal saline via gavage for a period of 21 days, while the lead group received 0.2% lead acetate through the same method. Following a two-week interval post-treatment with lead acetate or normal saline, the modified SPS model was applied to animals within the lead and SPS+Saline groups respectively, and this regimen was sustained for an additional week.

Based on the results of the one-way analysis of variance (ANOVA), there was a significant difference in freezing between the groups (F (Karrari et al., 2012; Schneider et al., 2012) $= 52.484$, $p < 0.001$). The supplementary test further examined the differences between the groups. The SPS+Saline, and SPS+Pb groups were found to have significantly higher levels of freezing compared to the Sham group ($p< 0.01$). This indicates that both the SPS+Saline and SPS+Pb groups exhibited increased freezing compared to animals in the Sham group.

Additionally, the SPS+Pb group showed significantly higher levels of freezing compared to the SPS+Saline group ($p< 0.05$). This suggests that the animals exposed to lead acetate (SPS+Pb group) displayed even more freezing compared to the SPS+Saline group (Fig. 3). Based on these findings, it can be concluded that lead acetate has an intensifying effect on the formation of conditioned fear memory in the animal model of post-traumatic stress disorder (PTSD).

The animals were subjected to a 5-minute session in the FCS without any specific interventions, one day following the initial memory test. This experimental setup was designed to investigate the influence of lead acetate treatment on the retrieval of conditioned fear memory and subsequent processes. Analysis of the second test results, focusing on freezing, revealed a statistically significant difference among the groups (F (Karrari et al., 2012; Schneider et al., 2012)$=48.273$, $p < 0.001$). Both the SPS+Saline and SPS+Pb groups exhibited significantly higher levels of freezing compared to the Sham group ($p<0.01$). Furthermore, the SPS+Pb group demonstrated significantly greater freezing than the SPS+Saline group ($p<0.05$) (see Fig. 3). These findings suggest that animals with a PTSD model, administered lead acetate for a duration of 21 days, exhibited notably heightened consolidation and recall of fear memory as compared to both the SPS+Saline group.

The third test was conducted one day after the second test and replicated its procedures, aiming to assess the impact of lead acetate on the reconsolidation or extinction of conditioned fear memory. A statistically significant difference in freezing levels among the groups was observed (F (Karrari et al., 2012; Schneider et al., 2012) $=42.972$, $p < 0.001$). Notably, both the SPS+Saline and Pb groups exhibited significantly higher freezing levels compared to the Sham group ($p<0.01$). Furthermore, the SPS+Pb group demonstrated significantly higher freezing levels than the SPS+Saline group ($p<0.01$) (Fig. 3).

In addition to its positive effects on traumatic memory acquisition and retrieval, lead acetate also contributed to the reconsolidation, rather than extinction, of fear memory in the stressed rats. As a result, by increasing the duration of freezing, Pb acetate induces a stronger fear memory in animals that have experienced the modified SPS model.

The results of the second experiment

The purpose of conducting the second experiment was to investigate the neuroprotective effects of eugenol on the memory impairment induced by lead in a stress disorder animal model. To achieve this goal, simultaneously with the initiation of lead acetate administration, animals were treated with eugenol, and during the 21-day period of lead acetate exposure, they also received eugenol four times a week. On day 21 of the experiment (three weeks after treatment with lead acetate and Eugenol), animals were subjected to the modified single prolonged stress model to induce illness. One week later, a memory test was conducted on them.

On the 28th day, which is one week after inducing the disease, the first test of conditioned fear memory was conducted. On the 28th day, which is one week after inducing the disease, the first test of conditioned fear memory was conducted. The analysis revealed a significant difference in immobility levels among the different groups (F (Dignam et al., 2019; Jaako-Movits et al., 2005) $= 3.015$, $p < 0.01$). The SPS+Pb+EU 25 group had significantly less freezing than the SPS+Pb+Saline group ($p<0.01$). Animal immobility was not significantly affected by eugenol doses of 50 and 100. Treatment with a 100 dose of eugenol significantly increased immobility in the SPS+Pb+EU 100 group compared to the SPS+Pb+EU 25 group ($p<0.05$) (Fig. 4). Eugenol at a dose of 25 mg/kg prevented the formation of traumatic memory in SPS rats receiving lead acetate, whereas higher doses of eugenol had no effect on the memory formation process in these animals.

The animals were placed in the FCS device without any special treatment one day after the first test to observe the process of recalling the traumatic memory in them for 5 minutes. A one-way ANOVA reveals a significant difference between groups (F (Dignam et al., 2019; Jaako-Movits et al., 2005) $=8.858$, $p < 0.001$). The level of immobility in the SPS+Pb+EU 25 group was significantly lower than in the SPS+Pb+Saline group ($P<0.01$). Furthermore, immobility was significantly higher in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups than in the SPS+Pb+EU 25 group ($P<0.01$) (Fig. 4). Because eugenol at a dose of 25 mg/kg prevented the formation of traumatic memory, it was also effective in reducing conditioned fear memory recall. Increasing the eugenol dose in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups resulted in a significant increase in memory recall compared to the SPS+Pb+EU 25 group. Traumatic memory recall in the SPS+Pb+EU 50 group was significantly higher than in the SPS+Pb+Saline group. This shows that a dose of 50 mg/kg eugenol resulted in enhanced fear memory in PTSD model animals treated with lead acetate.

The third memory test was performed one day after the second test and was identical to it in order to investigate the effects of eugenol treatment on the memory processes in SPS rats and those receiving lead acetate. A one-way ANOVA reveals a significant difference between groups (F (Dignam et al., 2019; Jaako-Movits et al., 2005) $=5.1542$, $p < 0.01$).

The level of immobility in the SPS+Pb+EU 25 group was significantly lower than in the SPS+Pb+Saline group ($P<0.01$). Furthermore,

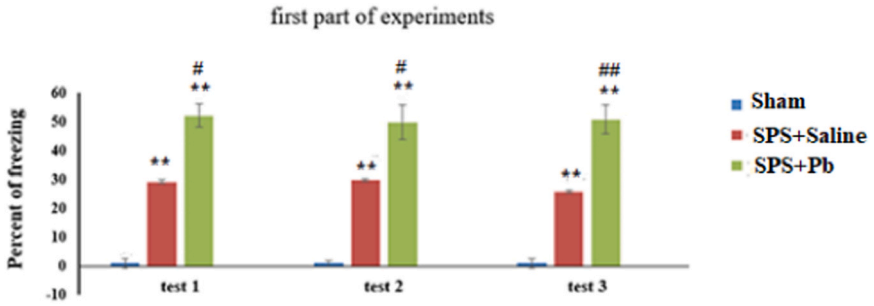

Fig. 3. Comparison of freezing in three memory tests between Sham, SPS+Saline and SPS+Pb groups. Data are presented as mean ± SEM. (n=10), ** ($p<0.01$) compared to the Sham group, # ($p<0.05$) compared to the SPS+Saline group, ## ($p<0.01$) compared to the SPS+Saline group.

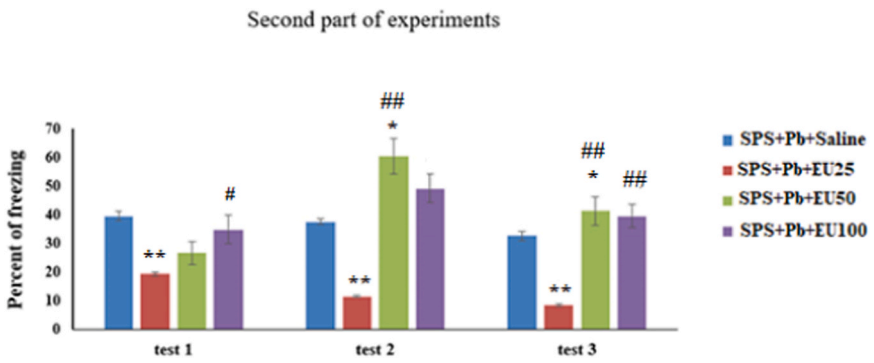

Fig. 4. Effects of different doses of eugenol on the effects of lead in memory process in post-traumatic stress disorder model animals. Data are presented as mean ± SEM. (n=10). ** (P<0.01) compared to the SPS+Pb+Saline group, #(P<0.05) compared to SPS+Pb+EU 25 group and ## (P<0.01) compared to SPS+Pb+EU 25 group.

immobility was significantly higher in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups than in the SPS+Pb+EU 25 group (P<0.01). The SPS+Pb+EU 50 group animals were significantly more immobile than the SPS+Pb+Saline group (P<0.05) (Fig. 4). Eugenol at a dose of 25 mg/kg resulted in inhibition of fear memory formation, as evidenced by a significant reduction in immobility in the third memory test compared to the SPS+Pb+Saline group. Increasing the eugenol dose in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups resulted in a significant increase in immobility compared to the SPS+Pb+EU 25 group. The immobility rate in the SPS+Pb+EU 50 group was significantly higher than that in the SPS+Pb+Saline group, indicating that traumatic memory was enhanced in animals receiving the 50 mg dose of eugenol. A dose of 50 mg/kg eugenol enhanced fear memory in stressed rats given lead acetate, an effect opposite to that of 25 mg/kg eugenol.

Results of weight analysis

Based on the data analysis of the first experiment, there is a significant difference in animal weight changes between the groups. The statistical analysis indicates that the effect is statistically significant (F (Karrari et al., 2012; Antonio et al., 2003) = 59.968, p < 0.0001). Comparing the average weight gains, it can be observed that the sham group gained an average of 41 g, the SPS+Saline group gained an average of 50 g, and the lead acetate group gained only 2.5 g (Fig. 5). These results suggest that exposure to lead acetate may have a negative impact on weight gain in animals compared to the sham and SPS+Saline groups. However, further interpretation of these findings would depend on additional contextual information and the specific objectives of the experiment.

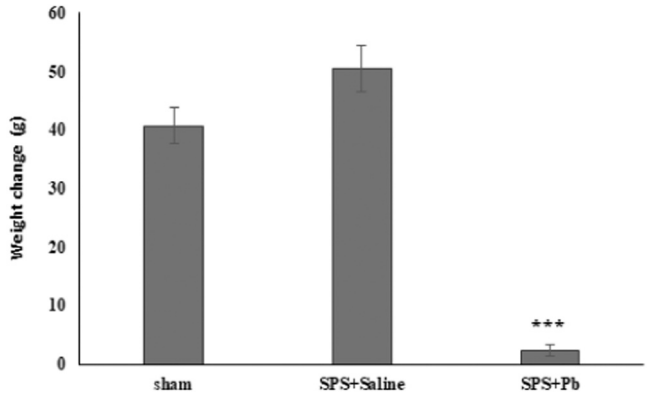

Fig. 5. Weight changes from day 1 to day 28 for animals in different groups of the first experiment. Data are presented as mean ± SEM.(n=8–10), *** (p<0.01) compared to the Sham group.

The second experiment investigated the weight changes of SPS rats that were treated with lead acetate and three different doses of eugenol over a period of 21 days. Data analysis revealed a significant difference between the groups (F (Dignam et al., 2019; Jaako-Movits et al., 2005) = 6.340, p < 0.0001), indicating that the treatments had an impact on the animals' weight. Specifically, the SPS+Pb+EU 25 group, which received a dose of 25 units of eugenol, showed a significant increase in weight compared to the SPS+Pb+Saline group (p < 0.01). The average weight gain for the SPS+Pb+Saline group was approximately 4 g, while the SPS+Pb+EU 25 group gained around 16 g during the experiment (Fig. 6).

Furthermore, when comparing the different eugenol dosage groups, the SPS+Pb+EU 25 group demonstrated a significant weight increase compared to the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups (p < 0.0001). In contrast, the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups experienced minimal weight gain, with less than one gram on average.

These findings suggest that chronic treatment with a 25-unit dosage of eugenol improved the normal growth retardation observed in SPS rats that were given lead acetate.

Results of open field test

The effects of lead on the number of crossings in the open field test and on the locomotor activity of the animals were measured. One-way analysis of variance showed no significant effect of treatment on the number of squares crossed. The data are not shown.

Qualitative observations

During the 31-day experimental period, notable disparities were

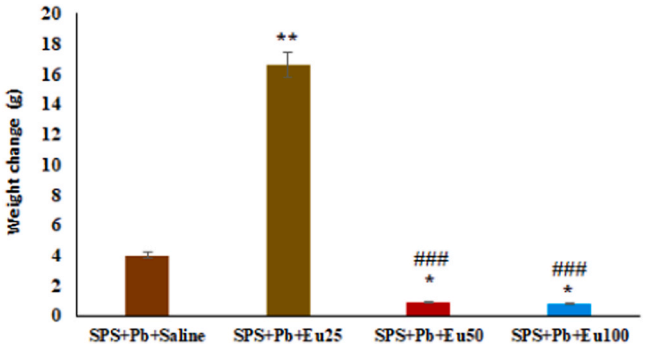

Fig. 6. Weight changes from day 1 to day 28 for animals in different groups of the second experiment. Data are presented as mean ± SEM. (n=8–10), * (p<0.05) compared to the Sham group, ** (p<0.01) compared to the Sham group, ### (p<0.0001) compared to the SPS+Pb+EU25 group.

observed among the animal groups, particularly in relation to those exposed to chronic lead acetate. The divergence in behavior exhibited by these animals was particularly prominent, with certain symptoms manifesting as early as 24 hours following the commencement of lead gavage. The ensuing symptoms were as follows:

1. Alterations in fecal characteristics: In comparison to the SPS+Saline group, the experimental subjects exhibited dry and black feces resembling constipation commonly observed in animals.

2. Decreased appetite and reduced daily water and food intake: As previously mentioned, there was a slight increase in body weight among the animal cohort after 21 days of weight measurement, despite a decrease in their overall appetite and consumption of water and food.

3. Diminished activity levels and lethargy: The experimental subjects displayed reduced physical movement and increased lethargy when compared to the SPS+Saline group.

In the second experiment, all animals exhibited symptoms of Post-Traumatic Stress Disorder (PTSD) and were administered lead acetate on a daily basis for 21 consecutive days. The animals were divided into four groups: one group received saline, while the other three groups received eugenol at intraperitoneal doses of 25, 50, and 100 mg/kg respectively.

Interestingly, despite being exposed to lead from the second week onwards, the group that received a dosage of 25 mg/kg of eugenol did not experience weight loss. However, the groups that received dosages of 50 mg/kg and 100 mg/kg of eugenol reported experiencing lethargy for approximately thirty minutes following the injection. In addition, these groups occasionally manifested a state resembling unconsciousness.

Discussion

Investigating the Effects of Lead on Memory Impairment in Post-Traumatic Stress Disorder: The prevalence of industrial and vehicular pollution has led to the introduction of certain scarce substances, such as lead, into the body, which exert unfavorable effects on the physiological system and organs of the body (Shvachiy et al., 2023). Lead has been found to have a detrimental effect on the central nervous system (Antonio et al., 2003). Conversely, the modern stressful lifestyle contributes to the emergence of numerous mental disorders, with Post-Traumatic Stress Disorder (PTSD) being one of the most prevalent. PTSD is characterized by a range of physiological abnormalities. In individuals affected by this condition, the inability to suppress traumatic memories significantly contributes to the initiation and worsening of symptoms associated with the disorder (Bremner, 2006; Orr and Roth, 2000).

Conditioned fear memory, once formed by the fear conditioning paradigm, is modulated by re-exposing rats to a shock chamber. Re-exposure reactivates the memory, leading to reconsolidation or extinction of the fear memory. Brief re-exposures to the context without a shock tend to induce reconsolidation, whereas more prolonged re-exposures induce extinction. Previous studies of contextual fear memory have shown that several parameters are involved in determining the fate of the recalled memory, including the characteristics of the shock during conditioning, the age, and the strength of the memory. It stands to reason that older, stronger memories require longer exposure to the context in order to reconsolidate. Studies have shown that exposure to the context without a shock for 5 minutes or less usually leads to reconsolidation, and exposure for 20 minutes leads to extinction of strong memories. Certainly, there is no clear threshold that can distinguish between the parameters that lead to reconsolidation and extinction (Yamada et al., 2009; Cassini et al., 2017).

In the first experiment of the present research, the animals of the SPS+Saline and SPS+Pb groups were placed for 5 minutes in the same cage where they had received a shock, without receiving another shock on the 21st day of the experiment. During this time, the conditioned fear memory is retrieved and put on the path of reconsolidation or extinction. In the second and third tests, which are exactly the same as the first test and are administered on the next two consecutive days, the process of recall and reconsolidation or extinction is repeated. The percentage of freezing in these three tests will indicate the path of the memory toward reconsolidation or extinction. If the amount of freezing does not decrease in successive tests, it means reconsolidation, or in other words, the previous memory is not undergoing extinction.

The first experiment of this study was designed to assess the effects of environmental lead contamination on the memory capacity in PTSD. Specifically, it aimed to investigate how exposure to a polluted environment would affect memory. The freezing levels of animals in three experimental groups—SPS+Saline, SPS+Pb, and sham—were measured in three consecutive memory tests. Animals that received lead acetate through gavage for a 21-day period displayed enhanced acquisition of traumatic events and memory retention. Lead acetate facilitated the acquisition of conditioned fear memory. Evidence supporting this claim was observed through an increase in freezing levels during the 5-minute assessment in the first experiment. Memory that is acquired as a result becomes consolidated into a strong memory trace and can be retrieved (Squire et al., 2015). The results of the second test, conducted 24 hours after the first test, demonstrated the persistence of lead's effects on the consolidation process and subsequently, memory retrieval. Animals chronically exposed to lead acetate also exhibited higher immobility levels compared to the SPS+Saline group in the third test, indicating the profound impact of lead on the fear memory in the PTSD model. In animals from the SPS+Saline group, which were affected by PTSD but not subjected to any treatment, freezing levels were around 30%, while in the lead-exposed group, freezing levels were approximately 60%. Overall, lead acetate had significant effects on all stages of memory, acting as an enhancing factor in fear memory in the stress disorder model.

The effects of lead acetate vary depending on the duration of exposure, available levels of lead, age of the individual, type of memory, and numerous other factors (Schneider et al., 2012). Lead acetate has different effects on learning and memory processes in childhood and adolescence, and exposure to low levels of lead during brain development affects the central nervous system function and disrupts neuronal evolution (Mycyk and Leikin, 2004). Lead increased learning in both males and females, but the avoidance and escape behaviors of female mice were greater than those of male mice. Pb-treated rats learned the active avoidance test better than control rats, not because of intellectual capacity, but because of altered and increased sensitivity to the foot shock stimulus. Perinatal lead exposure has been shown to disrupt emotion-regulating neural systems in the limbic system, resulting in sensory amplification of noxious stimuli similar to the model of post-traumatic stress disorder (PTSD) with anxiety-like symptoms (Ayaz et al., 2021). At high or low consumption levels, it has different impacts on memory. Previous studies also confirm the differential performance of various levels of lead on memory (Ramírez Ortega et al., 2021; Ferlemi et al., 2014).

Some studies at the cellular level have reported that lead replaces calcium as a secondary messenger in neuronal cells and blocks voltage-dependent calcium channels, thereby inhibiting calcium influx and neurotransmitter release. This action leads to the suppression of synaptic transmission, and on the other hand, lead can disrupt calcium-dependent processes such as learning, memory, neuronal growth and differentiation, and motor function. Additionally, it plays a role in causing impairments in learning, intelligence, memory, hyperactivity, cognition, behavioral problems, emotional disturbances, motor skills during childhood, reading and mathematical skills, and aggression (Ramírez Ortega et al., 2021; Rădulescu and Lundgren, 2019; Rocha and Trujillo, 2019).

In line with the present investigation, a study conducted on young Sprague-Dawley rats demonstrated that chronic exposure to lead resulted in impaired extinction of conditioned fear memory (McGlothan et al., 2008). Another study has also emphasized the disruptive effects of

lead on context-dependent fear memory and attributed this effect to a reduction in neurogenesis in the hippocampus (Jaako-Movits et al., 2005).

One of the potential mechanisms in the effects of lead on memory is oxidative stress. Lead toxicity induces oxidative stress in various brain structures, leading to memory impairment (Flora et al., 2012). Other studies emphasize the existence of multiple mechanisms of lead action (Silbergeld, 1992). Although the mechanism of lead effects was not examined in the present study, it is not unlikely that chronic lead consumption in patients with PTSD, who are susceptible to oxidative stress conditions in brain structures such as the hippocampus and are undergoing apoptosis, may act as an enhancer for these factors.

Despite numerous studies that have evolved in the field of lead effects on memory during different evolutionary periods, there is no study on the effects of lead on the various disorders of PTSD.

Investigation of the effects of Eugenol on memory in the PTSD model

The protective effects of Eugenol on the memory impairment caused by lead in the memory consolidation process were examined in animals with the PTSD model in the second experiment. Accordingly, along with the initiation of a 21-day exposure to lead acetate in animals with the PTSD model, they were also subjected to treatment with Eugenol (4 times a week). Eugenol was used in three doses. Different stages of memory were evaluated during three consecutive tests on three separate days. The effects of Eugenol were dose-dependent. The dose of 25 mg/kg significantly reduced immobility in SPS rats receiving lead acetate (first test of the first experiment) and prevented the acquisition of conditioned fear memory. In other words, it prevented the formation of traumatic memory in these animals. This effect was not observed at higher doses of Eugenol. Doses of 50 and 100 mg/kg were unable to have a significant impact on the memory formation process in these animals.

The recall of the traumatic memory was tested one day after the first test and again during the second test. The significant reduction in immobility in the SPS+Pb+EU 25 group, compared to the SPS+Pb+Saline group, indicates that eugenol at a dose of 25 mg/kg is effective in reducing conditioned fear memory recall and preventing traumatic memory recall. However, immobility was significantly higher in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups than in the SPS+Pb+EU 25 group. In other words, increasing the eugenol dose in the SPS+Pb+EU 50 and SPS+Pb+EU 100 groups increased memory recall. Traumatic memory recall was significantly higher in the SPS+Pb+EU 50 group animals than in the SPS+Pb+Saline group, indicating that the 50 mg/kg eugenol dose has resulted in an exacerbation of fear memory in stressed rats receiving lead acetate.

As expected from the results of the first and second tests, in the third test, Eu25 rescues the effect of Pb on enhancing fear memory in animals with stress disorders. A dosage of 25 mg/kg of Eugenol resulted in inhibition of fear memory formation, while increasing the dosage in groups SPS+Pb+EU 50 and SPS+Pb+EU 100 had the opposite effect.

In line with the findings of this study, previous studies have repeatedly demonstrated the effects of Eugenol on memory. It has been reported that Eugenol can enhance memory performance, neurogenesis, and dendritic arborization in the DG and CA1 regions of the hippocampus in mice (Akbar et al., 2021). Additionally, it has been reported that Eugenol can induce brain-derived neurotrophic factor (BDNF) in the hippocampus (Aryanezhad et al., 2021). This protein has the ability to increase the number of neuronal synapses and promote dendritic arborization, thus playing a role in neuronal plasticity and memory function (Akbar et al., 2021).

In the present study, the neuroprotective effect of Eugenol against lead acetate was observed. This neuroprotective role of Eugenol has also been demonstrated in previous studies with different doses and against various other neurotoxic compounds. Eugenol has been reported to reduce spatial memory impairment (dependent on the hippocampus) caused by scopolamine consumption (Garabadu and Sharma, 2019a). It has been reported that Eugenol improves behavioral disorders induced by the Parkinson's-inducing agent, 6-hydroxydopamine, at a dose of 10 mg/kg. In this report, the efficacy of Eugenol is attributed to its antioxidant role (Moreira Vasconcelos et al., 2020). The neuroprotective effect of eugenol (dose 50 mg/kg) ameliorates dementia and brain inflammation caused by ALCL3, a drug model of Alzheimer's disease (Zhao et al., 2019).

Eugenol acts as a neuroprotective agent in the brain of rats against aluminum-induced neurotoxicity through its water-repellent, antioxidant, anti-apoptotic properties, and also its neurotrophic ability (Said and Mokhtar, 2017). In other studies, the therapeutic effect of eugenol has been associated with its antioxidant and free radical scavenging capacity in improving cognitive and motor impairments caused by traumatic brain injury in rats (Barot and Saxena, 2021).

Researchers' studies have confirmed the obtained results by reporting dose-dependent effects of eugenol. In the Y-maze and MWM behavioral tests, eugenol (25 and 50 mg/kg) reduced scopolamine-induced lesions in spatial memory, learning, and memory in rats. However, the 12.5 mg/kg dose was ineffective (Garabadu and Sharma, 2019b). There are several mechanisms of action that support eugenol's neuroprotective effects, and further research is needed to substantiate this claim (Ulanowska and Olas, 2021).

In conclusion, our results show that chronic exposure to lead acetate exacerbates fear memory in stressed rats. That is, it increases the acquisition, retrieval, and consolidation of traumatic memories. A dose of 25 mg/kg eugenol ameliorated the effects of chronic lead acetate exposure in stressed rats. Eugenol 25 rescues the effect of Pb on enhancing fear memory, possibly due to its neuroprotective properties, and attenuated traumatic memory.

CRediT authorship contribution statement

Maryam Rabiei Golmakani: Investigation, Funding acquisition, **Kataneh, Abrari:** Methodology, Supervision, Data curation, Project administration, **Iran Goudarzi**: Conceptualization, Consultation, **Adeleh Khodaparast**, Writing – original draft preparation, Investigation, **Farzaneh Bagheri:** Writing – original draft preparation, Investigation.

Declaration of Competing Interest

None.

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