ผลของสารเฮสเพอริดินต่อความจำบกพร่องในหนูแรทที่ถูกเหนี่ยวนำด้วยเมโธเทรกเซท

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Effects of Hesperidin on Memory Impairments Induced by Methotrexate in Adult Rats

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หลักการและวัตถุประสงค์: เมโธเทรกเซท (methotrexate) เป็นยาเคมีบำบัดที่ใช้ในการรักษาโรคมะเร็งหลายชนิด การศึกษารายงานว่าเมโธเทรกเซท มีผลเป็นพิษต่อระบบประสาท เหนี่ยวนำให้เกิดภาวะความจำบกพร่อง เฮสเพอริดิน (hesperidin) เป็นสารธรรมชาติ มีคุณสมบัติลดการอักเสบและต้านอนุมูลอิสระ นอกจากนี้เฮสเพอริดินยังมีฤทธิ์กระตุ้นการเรียนรู้และความจำ ดังนั้นการวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของเฮสเพอริดิน ต่อความความจำบกพร่องที่เกิดจากเมโธเทรกเซท

วิธีการศึกษา: หนูแรท เพศผู้ จำนวน 24 ตัว สายพันธุ์ Sprague Dawley อายุ 4-5 สัปดาห์ ถูกแบ่งออกเป็น 4 กลุ่ม กลุ่มล่ะ 6 ตัว กลุ่มควบคุม (vehicle group) ได้รับน้ำเกลือและโพรพิลีนไกลคอล กลุ่มเฮสเพอริดิน (hesperidin group) ได้รับเฮสเพอริดิน กลุ่มเมโธเทรกเซท (methotrexate group) ได้รับเมโธเทรกเซท และกลุ่มเมโธเทรกเซทร่วมกับเฮสเพอริดิน (methotrexate+hesperidin group) ได้รับเมโธเทรกเซทร่วมกับเฮสเพอริดิน โดยเมโธเทรกเซทถูกฉีดให้กับหนูกลุ่มเมโธเทรกเซท และกลุ่มเมโธเทรกเซทร่วมกับเฮสเพอริดิน ในปริมาณ 75 มิลลิกรัม/กิโลกรัม ทางหลอดเลือดดำในวันที่ 8 และ 15 ของการทดลอง และได้รับเฮสเพอริดินทางปาก เป็นเวลา 21 วัน หลังจากสิ้นสุดการให้สาร 3 วัน หนูถูกทดสอบความจำโดยการทดสอบ novel object location และ novel object recognition

ผลการศึกษา: ผลการศึกษา พบว่าการทดสอบ novel object location และ novel object recognition ค่าระยะเวลาการ

Background and objectives: Methotrexate is a chemotherapy drug used to treat many different cancers. Several studies have reported that methotrexate has neurotoxic effects, leading to memory impairments. Hesperidin is a natural compound which exhibits anti-inflammatory and antioxidant properties. Furthermore, hesperidin enhances learning and memory. Therefore, the aim of this study was to investigate the effect of hesperidin on memory impairment caused by methotrexate.

Methods: Twenty-four male Sprague Dawley rats (age: 4-5 weeks) were divided into 4 groups (6 animals/group). Vehicle group received saline and propylene glycol. Hesperidin group received hesperidin only. Finally, methotrexate group received only methotrexate and methotrexate+hesperidin group received both methotrexate and hesperidin. A single dose of methotrexate (75 mg/kg) was administered by intravenous injection on day 8 and 15. Hesperidin (100 mg/kg) was administered per oral for 21 days. Three days after the end of drug administration, memory was tested using the novel object location and novel object recognition.

Results: The results showed that total exploration

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สำรวจวัตถุทั้งหมดในแต่ละกลุ่มไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05) การทดสอบ novel object location พบว่าหนูในกลุ่มควบคุม กลุ่มเฮสเพอริดิน และกลุ่มเมโธเทรกเซทร่วมกับเฮสเพอริดิน สามารถแยกความแตกต่างระหว่างวัตถุในตำแหน่งใหม่และตำแหน่งเก่าได้อย่างมีนัยสำคัญทางสถิติ (p<0.05) และเมื่อทำการทดสอบ novel object recognition พบว่าหนูในกลุ่มควบคุม กลุ่มเฮสเพอริดิน และกลุ่มเมโธเทรกเซทร่วมกับเฮสเพอริดิน สามารถแยกความแตกต่างระหว่างวัตถุใหม่และวัตถุเก่าได้ ในขณะที่กลุ่มเมโธเทรกเซทไม่สามารถแยกความแตกต่างระหว่างวัตถุใหม่และวัตถุเก่าได้ อย่างมีนัยสำคัญทางสถิติ (p<0.05)

สรุป: การศึกษาในครั้งนี้ชี้ให้เห็นว่าเฮสเพอริดิน สามารถป้องกันและฟื้นฟูความจำบกพร่องที่ถูกเหนี่ยวนำจากเมโธเทรกเซทในหนูแรทโตเต็มวัย

คำสำคัญ: เฮสเพอริดิน, เมโธเทรกเซท, ความจำบกพร่อง

time was not significantly different among groups in both novel object location and novel object recognition tests (p>0.05). In novel object location test, rats in vehicle, hesperidin and methotrexate+hesperidin groups could significantly discriminate between the objects placed in the novel and familiar locations (p< 0.05). Similar, in the novel object recognition test, rats in vehicle, hesperidin and methotrexate+hesperidin groups could significantly discriminate between the novel and familiar objects, whereas rats in methotrexate group did not discriminate between the novel and familiar objects (p<0.05).

Conclusion: The results of this experiment indicated that hesperidin is able to prevent and improve the memory impairments induced by methotrexate in adult rats.

Keyword: Hesperidin, Methotrexate, Memory impairment

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Introduction

Chemotherapy is the most importance for cancer treatment and increases survival rate of patients[1-3]. However, the treatment induces memory impairment, which decreases concentration, speed of information processing and memory[4]. Methotrexate is the first line therapy as of disease modifying antirheumatic drugs (DMARDs) for rheumatoid arthritis[5]. Furthermore, it is usually used in cancer treatments. Methotrexate inhibits the activity of dihydrofolate reductase (DHFR), which converts an inactive form of folic acid-dihydrofolate (DHF) into the active form-tetrahydrofolate (THF). Methotrexate is a powerful disruption of DNA synthesis[6]. However, neurotoxicity of methotrexate can be as a result of reactive oxygen species (ROS). Methotrexate causes an increase of apoptosis and significant reduction of hippocampal cell proliferation that lead to memory impairment[6-8]. Several reports have shown that side effects of methotrexate cause alterations of learning and memory[8-12]. Therefore, compounds with antioxidant properties may protect memory impairments from harmful effects of methotrexate.

Natural compounds such as flavonoids are very effective in neurodegenerative disease. Hesperidin, a flavonoid, is a part of natural flavanone group[13]. It can be found in citrus species, such as oranges, lemons and vegetables. Hesperidin exhibits various bioactivities, including anti-inflammatory, antioxidant defense mechanism and neuroprotective effects[13], [14]. It modulates neuronal signaling pathways, which involves in synaptic plasticity[14, 15]. In addition, hesperidin can enhance learning and memory[16] and it is potential in the treatment of neurodegenerative diseases[14]. Several evidences have shown that hesperidin (100 mg/kg/day) enhances cognitive and antioxidative stress[17].

Therefore, the aim of this study was to investigate the effect of hesperidin on memory impairments induced by methotrexate. The memory was tested using novel object location (NOL) and novel object recognition (NOR) tests.

Material and Methods

Animals and Experimental Procedures

Twenty-four male Sprague Dawley rats were 4-5 weeks old and 180-220 grams of body weight. The rats were provided from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The rats were adjusted to experimental conditions for a week period before the experiments. The rats were housed in standard laboratory conditions (25-28 °C temperature, 12 h light/12 h darkness). They were fed with food and water was supplied ad libitum. This study was approved by Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (ACUC-KKU-51/60).

Drug and treatment protocols

Methotrexate (pharmachemie BV, harsblem, Netherland) (75 mg/kg) was dissolved in 0.9% saline solution (Ajax Finechem Pty Ltd., Australia) for intravenous (i.v.) injection to the tail vein under anesthesia on day 8 and day 15. After methotrexate injections, leucovorin was administered to the rats by intraperitoneal (i.p.) injections at 18 hours (6 mg/kg), 26, 42 and 50 hours (3 mg/kg). The administration was similar to the application in patients in clinic. Leucovorin is clinically used to reduce cytotoxicity of methotrexate[18]. Hesperidin (ChemFaces Biochemical Co., Ltd., Wuhan, China) was dissolved in propylene glycol (Faces Biochemical Co., Ltd., China). Hesperidin was used to feed per oral (PO.) at a dose of 100 mg/kg/day at 10.00 a.m. for 21 days. Rats were randomly divided into 4 groups as follows (Fig 1):

Vehicle group: rats received saline solution (i.v.) and propylene glycol (PO.)

Hesperidin group: rats received 100 mg/kg of Hsd (PO.)

Methotrexate group: rats received 75 mg/kg of methotrexate (i.v.)

Methotrexate+hesperidin group: rats received 75 mg/kg methotrexate (i.v) and 100 mg/kg of hesperidin (PO.)

Behavioral testing

Novel object location (NOL) and novel object recognition (NOR) tests were performed in an open field black acrylic arena (50 cm × 50 cm × 50 cm). The arena was set up under an overhead digital camera connected to a computer tracking system (EthoVision®, XT Version 12, Noldus, Wageningen, Netherlands). The arena and objects were cleaned with 20% ethanol to eliminate any olfactory cues between trials and each experiment. The tests were adapted from Dix and Aggleton[19]. One day before NOL and NOR testing, the animals were habituated to explore environment of arena without objects for

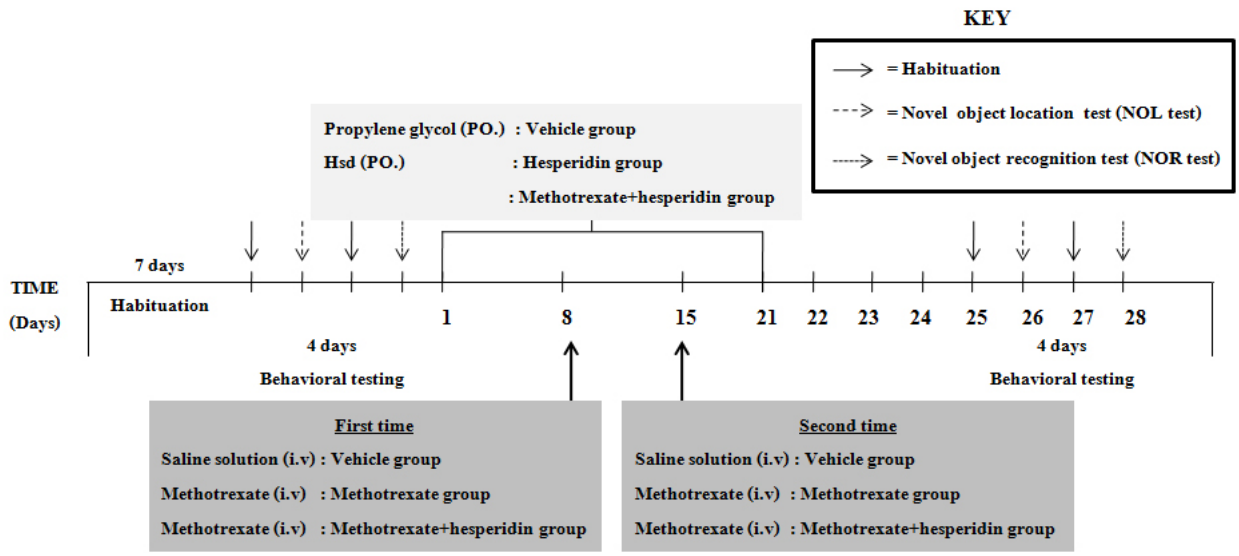

Figure 1 Experimental protocol: timeline of drug treatment and behavioral tests.

30 minutes and they were moved immediately to their home cages. After habituation, the rats were tested using NOL and NOR tests.

NOL test

Three days after the end of drug administration, NOL test was used to investigate spatial working memory. NOL test consisted of familiarization and choice trials. In the familiarization trial, rats were moved into the center of the arena and explored two similar objects placed in different locations (location A and B) for 3 minutes. The rats were moved from the area to their cages for 15 minutes. In the choice trial, the rats were returned to the arena to explore two objects for 3 minutes, one object was placed in the same location (familiar location), while the other object was moved to a new location (novel location).

NOR test

During the familiarization trial (1 day after habituation), the rat was placed into the center of the arena to explore two similar objects placed in different locations (object 1 and 2) for 3 minutes. Then, rat was returned to their home cages for 15 minutes. During the choice trial, one of the familiar objects and a novel object were put back to the same locations. The rats were returned to the arena and explored the objects for 3 minutes.

The object exploration in NOL and NOR tests were defined as the time that the rats spend exploring the objects by sniffing or touching within 2 cm.[19, 20]. The exploration time was recorded and used to calculate discrimination index (DI). DI is defined as the time difference between the novel and familiar locations or objects in the choice trail[21-24].

Statistical analysis

The data were expressed as mean + standard error of mean (SEM) using GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, CA, USA). Body weight was analyzed using two-way repeated measure analysis of variance (ANOVA). One-way ANOVA was used for comparisons among group of weight gain, total exploration time and DI. When ANOVA was significantly different, Bonferonni post hoc test was performed. One sample t-test was performed on DI whether the score differed from 0. A probability level of $p<0.05$ was accepted as statistical significance.

Results

1. Effect of hesperidin and methotrexate on body weight

Rat body weight was measured daily during the experiment as shown in Fig 2. Body weight of rats in methotrexate and methotrexate+hesperidin groups significantly was less than vehicle group ($p<0.001$, two-way repeated measure ANOVA), but not hesperidin group. After methotrexate injection, in methotrexate group, the body weight significantly reduced when compared with vehicle group on day 9 ($p<0.05$, two-way repeated measure ANOVA) and day 18 until day 21 ($p<0.001$, two-way repeated measure ANOVA). After day 9, rats in methotrexate group regained body weight. In addition, differences of body weight were significantly found between methotrexate+hesperidin and vehicle groups on day 18 to day 21 ($p<0.001$, two-way repeated measure ANOVA).

2. The improvement of hesperidin on memory impairment induced by methotrexate

2.1 NOL test

- NOL test before drugs administration

To assess memory function, a NOL test was carried out before three day of drugs administration. There was a no significant difference in the total exploration ($p>0.05$, one-way ANOVA, Fig 3A) and DI ($p>0.05$, one-way ANOVA, Fig 3B) among the four groups. Additionally, DI of rats in all groups had higher than 0 ($p<0.05$, one sample t-test, Fig 3B). The results indicate that rats in all groups could discriminate between two identical objects in different locations.

- NOL test three days after the end of the drug administration

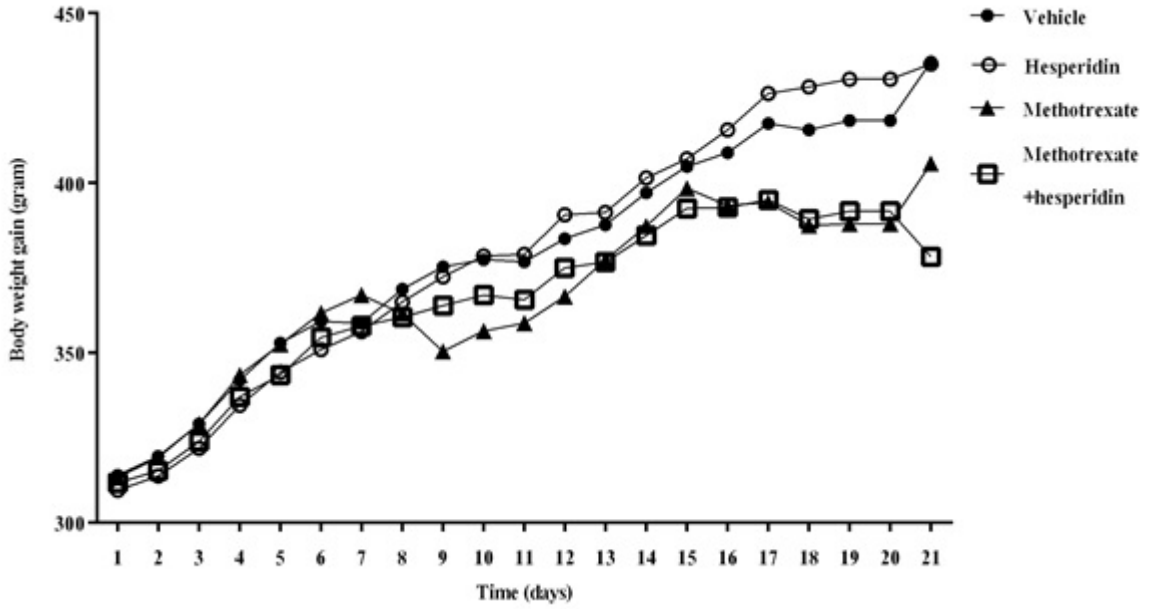

Figure 2 Body weight in vehicle, hesperidin, methotrexate and methotrexate+ hesperidin groups.

Three days after drug administration, the rats were examined by NOL test to investigate the effects of hesperidin on memory impairment induced by methotrexate. Total exploration time of all groups did not show significant difference ($p>0.05$, One-way ANOVA, Fig 4A). This result indicates that drug treatment did not affect locomotor activity. In addition, there were significant differences of DI among groups ($p<0.05$, one-way ANOVA, Fig 4B). Further analysis with Bonferroni Post hoc analysis reveals that DI of methotrexate group was significantly different when compared to vehicle, hesperidin and methotrexate+hesperidin groups ($p<0.05$, Fig 4B). DI of vehicle, hesperidin and methotrexate+hesperidin groups had a significantly higher than 0, but did not find in methotrexate group

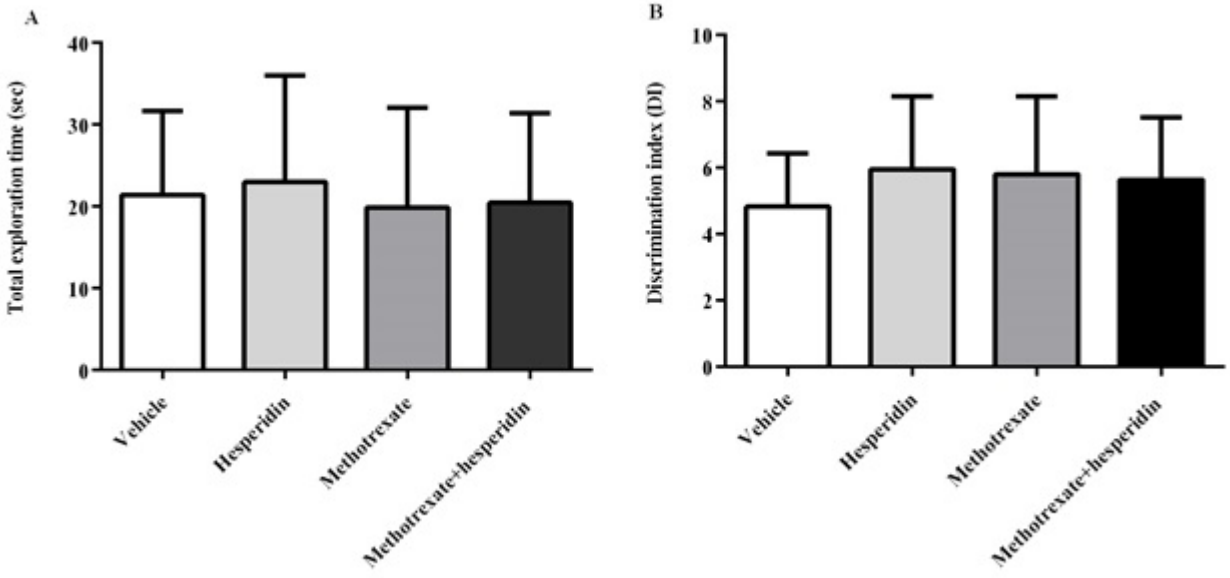

Figure 3 Total exploration times and discrimination index of the rats in the NOL test before treatment. Rats from all groups showed no significant difference in the total exploration time of the two objects ($p>0.05$, A). Discrimination index (DI) was not significantly different among groups ($p>0.05$, B). Data are given as mean±SEM.

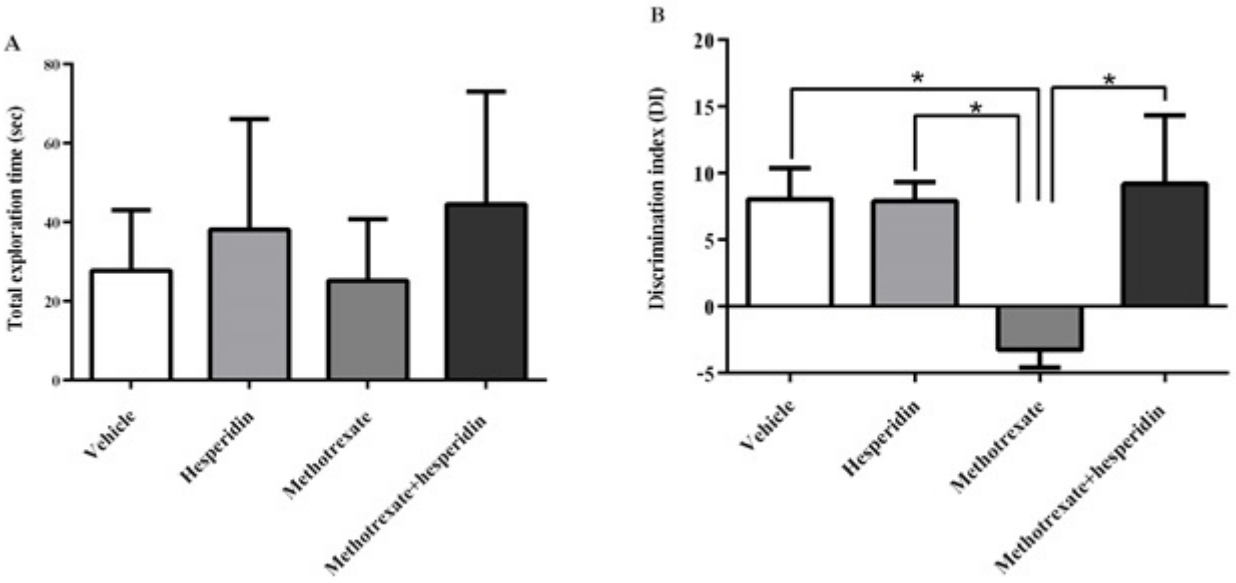

Figure 4 Exploration times of rats in NOL test after treatment. All data are expressed as mean exploration times (mean±SEM). Total exploration time was not significantly different among groups (p>0.05, A). Discrimination index (DI) of vehicle, hesperidin, and methotrexate+hesperidin groups was significantly different when compared with methotrexate group. The statistical analysis was calculated using one-way ANOVA with Bonferroni's post hoc test (*p< 0.05).

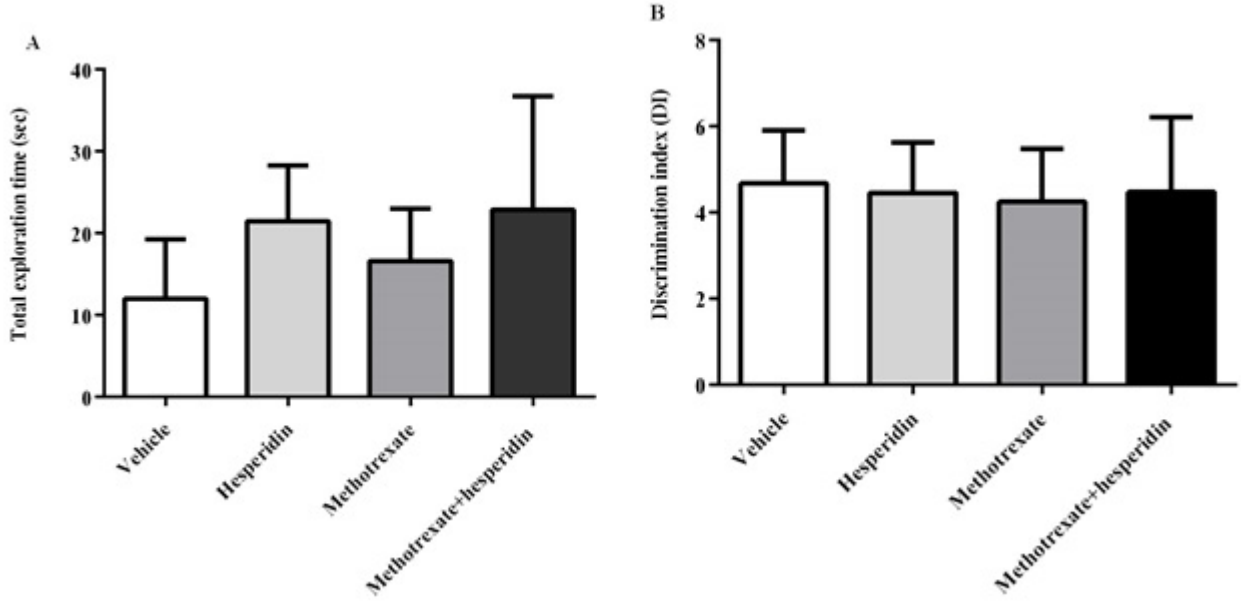

Figure 5 Total exploration time and discrimination index of the NOR before drug administration. In total exploration time, there were no significant differences among groups (p>0.05, A). DI of vehicle, hesperidin and methotrexate and methotrexate+hesperidin groups were not significantly different among groups (p>0.05, B). Data are given as mean ± SEM.

(p<0.05, one sample t-test, Fig 4B).

2.2 NOR test

- NOR test before drugs administration

Before drugs administration, the total there was no significant difference of the exploration time among four groups, indicating that rats in all groups had no impairment of locomotor activity to explore the objects (p>0.05; Fig 5A). The DI was not significantly different among groups (p>0.05; one-way ANOVA, 5B). Additionally, DI of rats in all groups had higher than 0 (p<0.05, one sample t-test, Fig 5B).

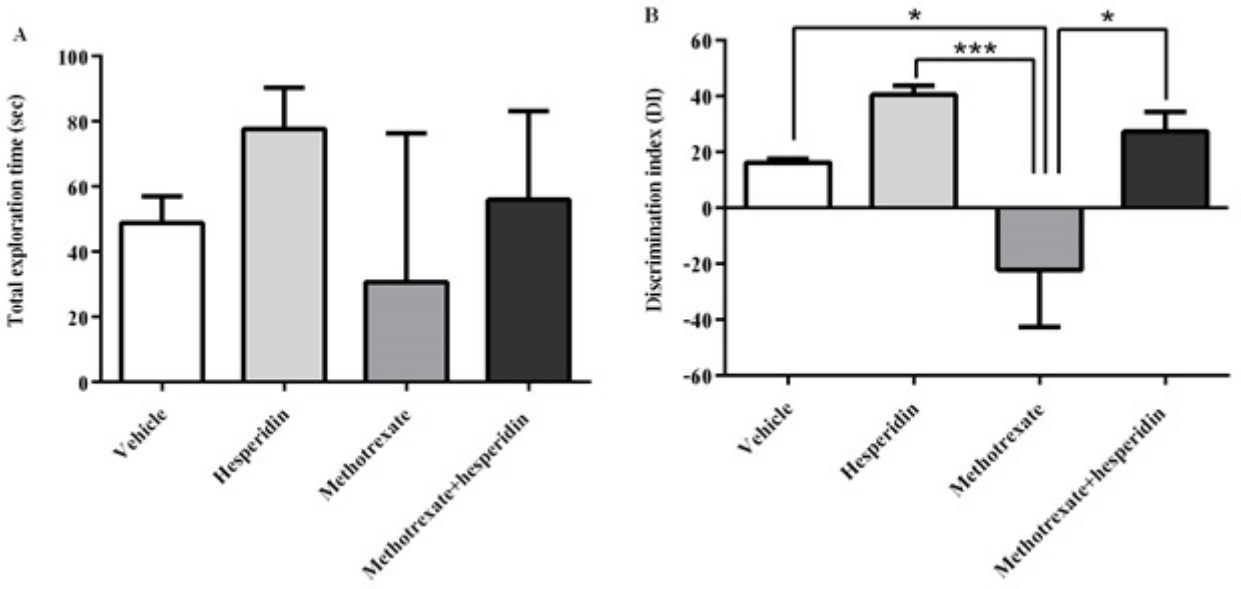

Figure 6 Total exploration times (A) and DI (B) in NOR test after drug. All data are expressed as mean exploration times (mean±SEM). The data analysis was performed using one-way ANOVA with Bonferroni's post hoc test (*p<0.05 and ***p<0.001, respectively).

These indicate that all rats were able to discriminate between the novel and familiar objects.

- NOR test after the end of the drug administration

There were no significant differences in the total exploration time among groups (p>0.05, one-way ANOVA, Fig 6A), which indicates no locomotor impairment. DI of rats in vehicle, hesperidin and methotrexate+hesperidin groups were significantly higher than 0, but did not find in methotrexate group (p<0.05, one sample t-test, Fig 6B). The data indicates that vehicle, hesperidin and methotrexate+hesperidin groups had the ability to discriminate between familiar and novel objects. However, the rats in methotrexate group did not discriminate between two objects. DI of methotrexate group was significantly different when compared to vehicle, hesperidin and methotrexate+hesperidin groups (p<0.05; p<0.001 and p<0.05, respectively; one-way ANOVA, Fig 6B), indicating their memory impairments.

Discussion

The present study investigated the effect of hesperidin on memory impairment after methotrexate treatment. During this study, methotrexate and methotrexate+hesperidin groups had a significantly less body weight than control group, whereas rats treated with only hesperidin did not show. The result indicates that methotrexate treatment induced decreases of body weight. This result is related to the mechanisms of methotrexate action, which stimulates DNA and RNA synthesis and induces apoptosis in both tumor and normal tissues. Gastrointestinal tissue is most frequently observed and may lead to intestinal mucositis symptoms, such as nausea, abdominal pain, and diarrhea[25]. It is the cause of decrease in body weight after methotrexate administration. Body weight of hesperidin group was not significantly different when compared with control group, indicating that hesperidin did not affect body weight which is consistent with a previous study[26].

In this study, both NOL and NOR tests were used for evaluated the memory impairment. Both tests require an intact adult neurogenesis in the SGZ of the dentate gyrus in the hippocampus. The hippocampus plays critical roles for the regulation of learning and memory[27]. The outcome of both tests, animals with no memory impairments will spend equal time exploring two objects in the familiarization trial, while spend long time to exploring the object

in the novel location or novel object in the choice trial. Previous studies have reported that methotrexate induces memory impairments[20, 28]. Similarly, results of the present study shows that rats treated with methotrexate (75 mg/kg) on days 8 and 15 could not discriminate between novel and familiar locations in NOL test. The NOR test, the rats treated with only methotraxate could not the discrimination a novel object from a familiar object. It indicates that animals receiving methotrexate showed memory impairments. Likewise, Yang and coworkers have confirmed that methotrexate enhances hippocampal dysfunction and cognitive impairment using novel object recognition (NOR) test[29]. Methotrexate negatively affects neurogenesis and induces memory impairments, which are related to DNA damage and/or deficiencies in DNA repair mechanism[30]. Additionally, methotrexate causes a reactive oxygen species (ROS) of the CNS, which may lead to neuronal toxicity, neuro-inflammation as well as neuronal cell death[8]. The present study shows that methotrexate+hesperidin groups significantly improved memory impairment as compared with methotrexate group. This was confirmed by DI in NOL and NOR test. In NOL test, rats in all groups were able to discriminate between two identical objects in novel and familiar locations. However, rats had received hesperidin for 21 days spent significantly more time exploring the object in the novel locations compared to methotrexate group. In addition, there was a significant difference in the DI between methotrexate and methotrexate+hesperidin groups. In the NOR test, the rats in all groups had no significant difference in the DI. After 21 days of drugs administered, rats receiving hesperidin was able to discriminate between novel and old object. The DI of methotrexate group was significantly reduced in comparison with hesperidine group, but rats treated with both methotrexate and hesperidin showed an increase of DI. This finding indicates that co-treatment with methotrexate and hesperidin improved memory impairments. Similarly, the administration of hesperidin (100 mg/kg) recovered memory deficits in the APPswe/PS1dE9 trangenic mouse model of AD[17]. Hesperidin can enhance neuronal function and induce neurogenesis by increasing blood flow[16]. As mentioned, hesperidin can cross into the brain via the blood brain barrier[31]. Therefore, the hesperidin can enhance memory.

In addition to memory impairments, methotrexate reduces antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)[32, 33], which result in increasing cellular damage and neurogenerative diseases. Hesperidin is one of the most important antioxidants, which can protect neurogenerative diseases. Hydroxyl groups of hesperidin donates electrons to the radicals to decrease ROS[34]. Similarly, Antunes and coworkers have found that hesperidin increases Ark/Nrf2 signaling and enhances levels of antioxidant enzymes, such as CAT, SOD, and GST in AD rat models[35]. Also, Antunes et al.[20] has reported that hesperidin shows neuroprotection against neurotoxicity induced by 6-hidroxidopamine (6-OHDA) in a Parkinson's disease (PD) rat model. These evidences support our findings that hesperidin improves memory impairments in methotrexate treated-rats.

Conclusion

Hesperidin (100 mg/kg) significantly improved memory impairments caused by methotrexate. Thus, the present study shows the evidence that hesperidin may be useful as a supplement to prevent and improve memory impairments.

Acknowledgments

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