European Review for Medical and Pharmacological Sciences 2019; 23: 9660-9666

Ameliorative effect of metformin on cyclophosphamide-induced memory impairment in mice

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Abstract. – OBJECTIVE: Cyclophosphamide (CYP) is a chemotherapeutic agent that is widely used as an adjuvant cancer treatment. Unfortunately, this drug is associated with secondary side effects, including cognitive impairment up to 70% of cancer survivors. The mechanism of this memory impairment is unclear. Thus, to understand the cognitive impairments caused by this chemotherapeutic agent, a clinically relevant dose to cancer treatment was used in mice to establish the chemobrain models, and the spatial memory of these mice was assessed using multiple behavior tests. In addition, metformin (MET) is widely used as an anti-diabetic drug and protects against oxidative stress and hepatotoxicity. Thus, this study tested the protective effects of MET in the chemobrain models.

MATERIALS AND METHODS: Four groups of mice, which weighed about 18-30 g, were collected and divided into 4 groups: control, CYP, MET, and CYP+MET groups. A 100 mg/kg dose of CYP was administered intraperitoneal (on alternate days) for a total of 4 doses. MET was dissolved in the mice's drinking water bottles at a 5 mg/ml concentration from day zero to the end of the treatment period. The mice's memory was tested using hippocampal-dependent tests, including the Y-maze, novel object recognition, and elevated plus maze tests. These tests were performed for three consecutive days after 24 h of the last dose of CYP.

RESULTS: The mice treated with CYP exhibited a decline in memory function in all the behavioral test studies, and this decline was significant in the Y-maze test. However, this decline was rescued by MET administration.

CONCLUSIONS: The clinically relevant model suggests that CYP treatment causes a decline in mice models spatial memory that might be improved by MET administration.

Key Words:
Metformin, Chemobrain, Cyclophosphamide, Cognitive impairment.

Introduction

Despite efforts to improve chemotherapeutic cancer treatments over the years, the survival rate and quality of life in advanced cancer has increased[1]. However, cancer survivors are left with many side effects, such as hepatotoxicity, nephrotoxicity, cardiotoxicity, and cognitive impairment[2,3]. Chemotherapy-induced memory impairment (chemobrain) is one side effect of chemotherapy reported by patients who underwent cancer treatment and it affected up to 70% of cancer survivors[3]. Cyclophosphamide (CYP), a synthetic, broad-spectrum anticancer drug and alkylating agent, is an effective treatment for a wide range of cancers, including breast, myeloma, ovarian, neuroblastoma, and leukemia[4,5]. The active CYP mechanism binds to the DNA and inhibits DNA replication, initiating apoptosis[6]. However, the chronic administration of CYP may induce toxicity to other non-target tissues, resulting in undesirable effects, such as alopecia, nausea, fatigue, and cognitive impairment[7]. However, the exact mechanism and etiology that underlie the cognitive deficits caused by chemotherapy are unclear. Metformin hydrochloride (Metformin, MET) is an anti-diabetic agent in the biguanide class. MET, which is taken orally, is a first-line treatment for type 2 diabetes patients[8]. It reduces blood glucose by increasing insulin receptor sensitivity in peripheral tissues and inhibits neoglucogenesis in the liver[9]. The complete details about other mechanisms of MET's action are yet to be fully explored[10]. However, studies[11-13] show that MET can restore cognitive function in some conditions, such as oxidative stress, high-fat diet ingestion, and obesity. This study is designed to

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detect the MET's role in preventing the memory dysfunction induced by acute CYP treatment in cancer patients. Thus, this study is designed to establish a chemobrain model by treating mice with CYP, to induce cognitive impairment, and to investigate whether MET can rescue the memory impairment, which resulted from CYP treatment. The model's cognitive impairment is evaluated by using behavioral tests, such as the Y-maze, novel object recognition (NOR), and elevated plus maze (EPM) tests.

Materials and Methods

Chemicals

Cyclophosphamide and metformin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals

Forty mice (18-25 g) were individually housed and acclimatized to laboratory conditions for a week. The animals were maintained at 12 h light/dark cycle with a room temperature and relative humidity ranges of 25-28°C and 45-55%, respectively. Purified drinking water and pelleted rodent food were given *ad libitum*. The animals were observed for their wellbeing daily, and their body weight was measured before dosing. The study protocol was approved by the Qassim University Animal Ethics Committee (Approval ID: 2019-CP-6).

Drug Administration

After the acclimatization period, the mice were grouped into 4 groups (n = 10) namely, group 1: vehicle control, group 2: CYP, group 3: MET, and group 4: CYP+MET. The mice of group 2 and 4 were intraperitoneally (i.p.) injected with CYP (100 mg/kg) every 2 alternative days. Group 1 and 2 were provided purified drinking water throughout the study. Metformin (5 mg/ml) was admixed in the drinking water and given from a day before CYP treatment during the entire study period. After the animals received 4 scheduled CYP doses, they were subjected to behavioral tests. All the behavioral tests were performed during the light phase of the cycle with uniform lighting conditions (30 ± 2 lux). The animal wellbeing and body weight were monitored daily.

Assessment of Spatial Memory Using Y-Maze

The Y-maze test assesses the ability of the mice to recognize the places they have already explored and their propensity to explore new places[14]. The Y-maze was custom made for the mice using wood and had dimensions of 39.5 x 8.5 × 13 cm (l × b × h). Each of the 3 arms were at 120° to other arms and were painted brown to provide a smooth finish for easy disinfection between each animal's test. The apparatus was placed on a floor. A light was placed in the center and right above the top of the maze to ensure equal light distribution. A camera was used to record all the testing sessions. The Y-maze tested the mice's working memory; it measured the spatial memory functions of the CYP, MET, and CYP+MET treated mice, as well as the control mice.

The training sessions, which allowed the animals to freely explore two arms (the arm in which they were placed (start arm) and another arm (familiar arm) placed at either the left or right of the start arm), lasted 10 min. During the second session, which lasted approximately 5 min, the mice were allowed to explore the entire maze, including a new arm (novel arm). The time between the first and second sessions was 3 h. The second session was video recorded to identify the number of entries into and the time the mice spent in the novel arm. An arm entry was counted when more than half of their bodies entered any of the 3 arms. The time the mice spent in each arm was also recorded. The number of entries into and the time each mouse spent in the novel arm were scored and analyzed.

The Norwegian Tenecteplase Stroke Trial (NOR) Test

The NOR test is a behavioral test that measures hippocampal dependent memory[15]. The test apparatus was a cube with an open top made of wood. The dimensions of the box were 40 x 40 x 40 cm. The familiarization objects included 2 white teacups with handles, and the novel test object was a rectangular green metal box that had a similar size to the teacup. In this test, the mice were introduced to and allowed to explore 5 teacups for 5 min. Then, the animals were returned to their cages. Three hours later, the animals were tested for 10 min. One of the teacups was placed in the same position of the mice's previous exploration of the NOR apparatus, while the other object was replaced with the novel object. All the mice were returned to the test, and the time they spent

exploring the novel object was measured using a camera set above the apparatus with ample light, and the results were analyzed[16]. The time the mice spent within a predefined boundary for both the objects was calculated using a stopwatch.

The Elevated Plus Maze (EPM) Test

The EPM test is a behavioral test that is commonly used to measure the learning and memory processes. The EMP apparatus for the mice was acquired from Medicraft (Ponekkara, India). It consisted of two opposing arms. The open arm's length was 30 cm and width was 5 cm, while the closed arm's length was 30 cm and width was 5 cm. The height of the sidewalls was 15 cm. An open central area measured 5 cm^2, and the maze was elevated 30 cm above the floor.

During the acquisition trial, each mouse was individually placed at the end of the open arm, facing opposite the central platform. The mice were allowed to explore the apparatus for 5 min. After 3 h, each mouse was placed facing opposite the central platform. The latency time (LT) was recorded. The LT is the time it took a mouse to move from the end of the open arm and place all four of its paws inside either of the closed arms. A video camera was placed directly above the central platform. The experiment was conducted under dim light during the day[17].

Blood Glucose Test

The blood glucose test was used to obtain the mice's glucose levels. The submandibular bleed technique was used to obtain optimum-quality blood. An Accu-Chek glucometer with strips was used to test the mice's blood glucose based on the manufacturing instructions.

Statistical Analysis

The results are presented as group means ± S.E.M. and were analyzed using GraphPad Prism 5 software (San Diego, CA, USA). The Y-maze, NOR, EPM, and blood glucose data for each group were analyzed using unpaired two-tailed *t*-tests, and all the treatment groups' data were compared to the control group. A value of $p \leq 0.05$ was statistically significant.

Results

Behavioral Performance in the Y-Maze

A significant difference in the total time the CYP-treated mice spent in the novel arm compared to the controls was found, whereas the MET and CYP+MET time was not significantly different from the control group. Furthermore, the CYP+MET group spent more time in the novel arm than the CYP group, which reveals the potential protectant effect of MET when it is co-administer with CYP. In addition, the MET group and CYP+MET group both chose to enter the novel arm at the beginning of the Y-maze test session. However, significantly fewer ($p < 0.05$) animals from the CYP group entered the novel arm, indicating that the animals did not distinguish the novel arm from the other arms.

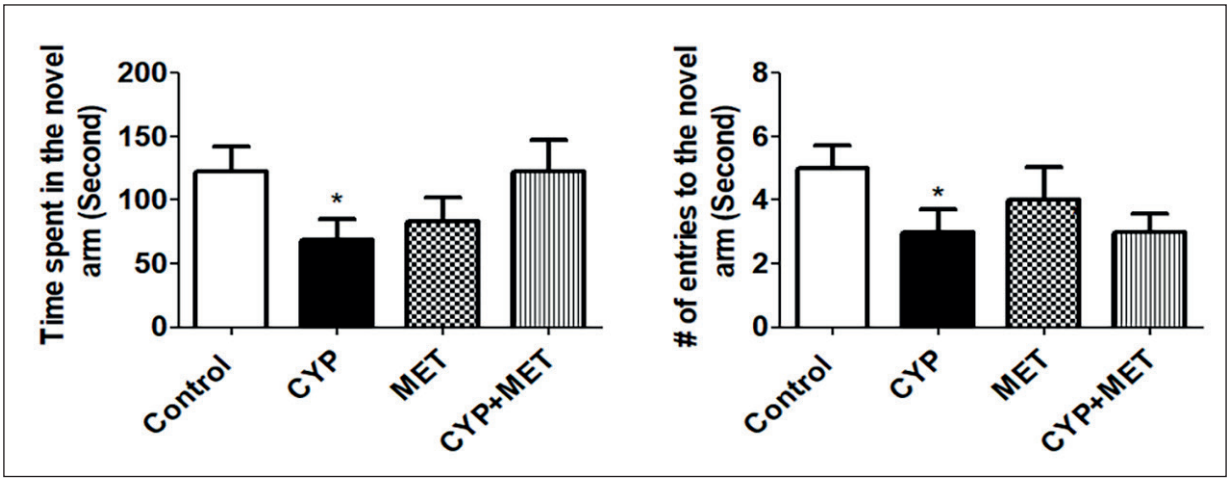

Figure 1. Y-maze test performance (n = 10/group). The CYP-treated mice showed cognitive deficits compared to the non-treated, control mice. The MET-treated mice experienced a partial reversal of these deficits. A statistically significant difference ($p < 0.05$) between the CYP-treated and control mice test results was identified, but no statistically significant difference was found in the time the CYP- and CYP+MET-treated mice spent in the novel arm.

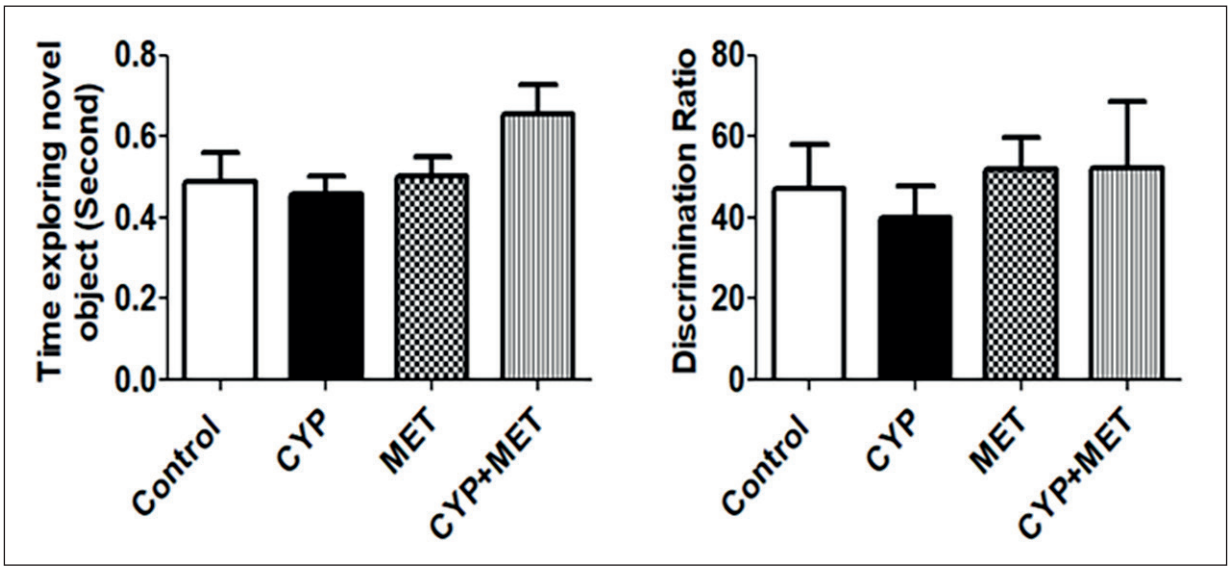

Figure 2. NOR test results. No significant difference in the treated and control groups' NOR test results was found.

Effects of MET Treatment on the NOR Test

No significant difference was found in all the group's (control, CYP, CYP+MET, and MET) NOR test results in either the training or testing sessions, which suggests that CYP, MET, and the combination of these drugs did not alter the chemobrain memory function in the treated mouse models (Figure 2).

Effect of MET and CYP on the Transfer Latency of Mice

In the CYP group (4 doses of CYP 100 mg/kg i.p. on alternate days), the LT increased on the 12th day, indicating memory impairment. The MET group (MET 5mg/ml of drinking water) experienced an increased LT on the 12th day of the experiment compared to the control group. Finally, the CYP+MET group (MET 5mg/ml in drinking water and 4 doses of CYP 100 mg/kg i.p. on alternate days) also experienced an increased L.T. on the 12th day. Therefore, MET had no effect on CYP-induced memory impairment (Figure 3).

Blood Glucose Test

To determine the animals' glucose-level sensitivity to the cytotoxic effects of CYP, the glucose levels of the animals in all four groups were evaluated following their treatment sessions. As shown in Figure 4, the CYP and MET groups did not experience a significant change in their glucose levels, indicating that CYP does not affect glucose levels during short-term treatments. However, a slight decrease in the glucose levels of the CYP- and CYP+MET-treated mice was observed, which may be due to a decrease in food intake as a result of the CYP treatment.

Discussion

The present work examined the effect of CYP on memory function using multiple behavioral tests. From the study it was found that MET

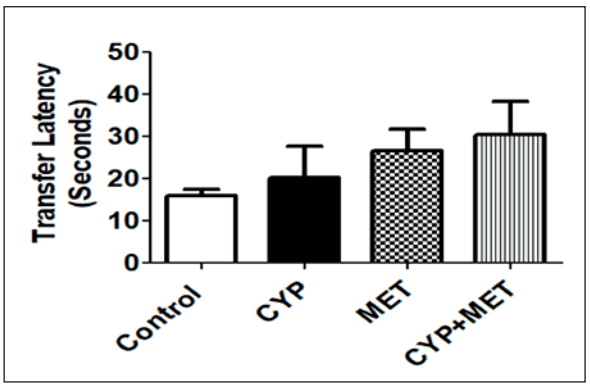

Figure 3. EPM test performance (n = 10). The CYP-, MET-, and CYP+MET-treated mice had higher transfer latency times than the non-treated, control mice. This test indicates a tendency toward memory impairment in the CYP, MET, and CYP+MET mice compared to the control mice even though this impairment is not statistically significant.

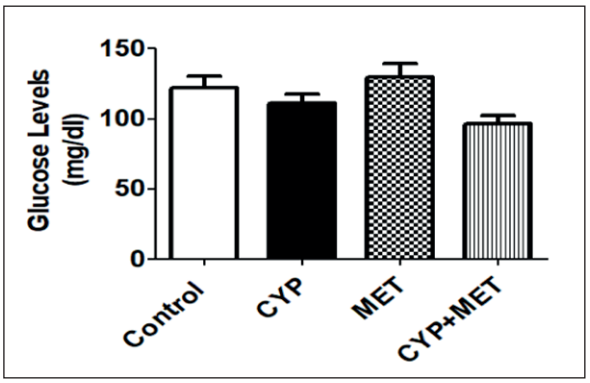

Figure 4. The effect of CYP, MET, and CYP+MET treatment on the blood glucose levels of chemobrain induced mice (n = 10).

might reduce the toxicity of chemotherapy[18]. Thus, the co-administration of MET and CYP might reduce the toxic effects of CYP.

Previous researches identified the beneficial effects of MET in hepatotoxicity, nephrotoxicity, and ischemic brain damage models[18,19]. The preventive administration of MET before chemotherapy treatment might reduce the adverse hepatotoxic and nephrotoxic effects of the drugs in rodent models[20,21]. Cognitive deficits in patients with cancer are associated with persistent neuro-inflammation[22,23]. In addition, neuropathic pain induced by chemotherapeutic agents is associated with a persistent increase in the expression of inflammatory markers in the brain[24]. Several studies reported that MET has anti-inflammatory effects that might ameliorate chemotherapy toxicity. In addition, MET enhances memory function and neurogenesis in diabetic patients[25], as well as experimental animal models[10,26]. In the current study, significant memory impairment in the CYP-treated mice was observed, and pre-treatment with MET to these mice improved the memory function.

The Y-maze is a hippocampal-dependent task that was chosen to test the experimental groups' spatial working memory. The results showed that the animals that received only MET were able to distinguish the novel arm from a familiar arm or start arm. Therefore, the findings for the MET group did not differ from the control group, indicating that the MET dose that was administered did not affect the mice's ability to complete this task. However, mice treated with CYP showed some degree of cognitive impairment. The CYP-treated mice spent less time in the novel arm and entered the novel arm fewer times than the control group. However, these cognitive deficits were improved when MET and CYP were co-administered. The cognitive impairments caused by CYP-treated mice were statistically significant, revealing that CYP treatment causes cognitive decline.

The present study also used a NOR task to assess memory function[16]. The animals' memory was not impaired during this task, suggesting that not all the animals' cognitive functions were affected by CYP treatment. Moreover, working and spatial memory are hippocampal-dependent tasks, whereas NOR tasks are dependent on the dorsal hippocampus[27]. The current study's results suggest that CYP treatment causes cognitive impairment. This impairment in memory function is not due to the animals' lethargy because the other tests had positive memory-loss results. The data suggest that CYP treatment might disrupt the memory that relies on intact hippocampal function and MET could fix these deficits.

The CYP-treated mice showed a decrease in the total time they spent in the open arms of the EMP test, and they significantly increased their freezing time, indicating that CYP results in anxiety-like behavior. Anxiety is a natural response that promotes adaptive survival through escape from unnecessary danger. However, too much anxiety may disrupt regular brain functions, reducing the behavioral activity necessary for adaptation. The amygdala plays a vital role in the expression of anxiety or fear, and the medial prefrontal cortex is important in the regulation of the amygdala-mediated expression of fear. This study evaluated the ameliorative effect of MET pre-treatment with CYP on the cognitive decline caused by CYP treatment. The results revealed that MET increased the animals' latency time in the EPM test, indicating that MET may incite anxiety in mice. In the current study, the latency time was not reduced through the co-administration of MET and CYP, indicating that MET does not protect against the anxiety caused by CYP.

Conclusions

Chemotherapy improves survival rate of cancer patients. MET reduces glucose levels and protects against the hepatotoxicity and nephrotoxicity caused by chemotherapeutic agents. This

study tested the protective effects of MET on the memory impairment induced by chemotherapy using chemobrain mouse models treated with CYP. The results of this study revealed that memory impairment occurred due to CYP treatment, and these impairments were rescued through the administration of MET. Therefore, MET could be used during chemotherapy to reduce the toxicity and cognitive impairments.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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