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Research Article
Metformin Induced Cognitive Impairment and Neuroinflammation in CMF-Treated Rats

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Abstract

Background and Objective: Metformin (MET) has been shown to reduce the toxicity and memory dysfunction caused by chemotherapeutic agents, thereby improving patient survival and quality of life. The current study aimed to evaluate the effects of MET treatment on the survival and cognitive function of rats receiving CMF chemotherapy (cyclophosphamide [CYP], methotrexate [MTX] and 5-fluorouracil [5-FU], a regimen for breast cancer treatment) for 2 weeks. **Materials and Methods:** Forty male rats were divided into four groups (n = 10 per group): control (saline); CMF group (2 mg kg^{-1} MTX, 50 mg kg^{-1} CYP and 50 mg kg^{-1} 5-FU weekly); MET group (2.5 mg mL^{-1} MET daily); and CMF+MET group (CMF [2 mg kg^{-1} MTX, 50 mg kg^{-1} CYP and 50 mg kg^{-1} 5-FU weekly] and 2.5 mg mL^{-1} MET daily). Animals were monitored daily to assess survival and their body weights were measured every 3 days. After treatment, cognitive function was evaluated via behavioural tests. CMF and CMF+MET treatment resulted in decreased survival and body weight compared with control and MET-treated rats, as well as impaired memory function as assessed by the Y-maze test. **Results:** Co-administration of MET alleviated the effect of CMF on survival, but it appeared to increase memory impairment according to the elevated plus-maze test. CMF+MET-treated rats showed significantly decreased blood glucose levels compared with controls. Rats treated with CMF+MET showed increased interleukin-6 expression in brain tissue compared with MET-only and CMF-only treated animals. **Conclusion:** While MET may improve survival during CMF treatment, it may harm cognitive function and increase neuroinflammation.

Key words: Metformin, chemotherapy, survival rate, mortality, Y-maze, novel object recognition, elevated plus maze

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many studies have shown that early diagnosis of cancer and chemotherapy treatment can increase survival and halt cancer progression[1]. Most chemotherapeutic agents act by inducing cytotoxicity, ultimately leading to cancer cell death. However, this toxicity is associated with acute and chronic side effects[2,3], such as cognitive impairment or "chemobrain"[4,5], which can range from moderate to severe. These impairments can affect the emotional, behavioural and mental status of patients[6,7]. The burden of cancer is increasing, with about 90 million people living with cancer worldwide[8]. Cognitive impairment affects up to 75% of patients undergoing chemotherapy and persists in 17-34% of cancer survivors[6]. Chemobrain remains clinically challenging; few therapeutic strategies are available to treat the neurotoxicity caused by chemotherapy.

Briones and Woods reported that CMF chemotherapy (the combined administration of cyclophosphamide [CYP], methotrexate [MTX] and 5-fluorouracil [5-FU], a common regimen for breast cancer) intraperitoneally (i.p.) administered to rats disrupted learning and memory processes[9]. Our previous study and other studies have concluded that CYP and/or doxorubicin can impair memory function in rodent models[4,5]. Furthermore, studies have shown that CYP can increase cytotoxicity, ultimately leading to apoptosis in both *in vitro* and *in vivo* models[5,10,11]. The mechanisms of chemobrain are not yet fully understood. It has been proposed that the mechanism may involve chemotherapy-induced hepatotoxicity, nephrotoxicity and cardiotoxicity[12-14]. Also, chemotherapy can affect several protein kinases that are important in the regulation of memory function. For example, experimental studies have indicated that the mammalian Target of Rapamycin (mTOR) is an important regulator of memory function and doxorubicin administration can inhibit mTOR protein expression and function[15].

Metformin (MET) is an antidiabetic drug belonging to the biguanide class of therapeutics that is mainly used to treat type 2 diabetes mellitus[16]. MET is well-known to exert its effect by increasing insulin receptor sensitivity and reducing hepatic glucose production. MET is reported to activate Adenosine Monophosphate (AMP)-activated Protein Kinase (AMPK)[17-19], which may affect the actions of other proteins such mTOR and Protein Kinase B (PKB, also known as Akt). Akt activation by AMPK causes increased trafficking of the glucose transporter to the cell surface, where it is involved in the cellular uptake of glucose to reduce glucose levels in the blood[20]. However, activation of AMPK leads to the inactivation of mTOR[21].

Previous research has suggested that MET may exert a protective effect during chemotherapy[5,12]. However, to the best of the authors' knowledge, the effect of MET on the side effects associated with CMF chemotherapy, such as cognitive impairment, has not been elucidated.

The objective of this study was to determine the effects of CMF treatment on the survival rate and cognitive function of rats and to assess the effects of MET co-administration.

MATERIALS AND METHODS

Study area: This research project was conducted from September-November, 2020 at the Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University, Kingdom of Saudi Arabia

Drugs: CYP (Endoxan®) was obtained from Baxter (Germany); MTX (Methotrexate®) was obtained from Hospira UK Ltd. (United Kingdom); 5-FU (Utoral®) was obtained from Korea United Pharm Inc. (South Korea) and MET hydrochloride (Metfor®) was obtained from Tabuk Pharmaceuticals (Saudi Arabia).

Animals and treatments: Forty male rats (10-12 weeks-old; 200-250 g body weight) were individually housed in a pathogen-free room with a 12 hrs light/dark cycle (lights were turned on at 6:00 am). The rats were given free access to food and water at all times during the 2-week study period. The animals were divided into four groups (n = 10 per group). The control group received two doses of saline by i.p. injection, weekly. The CMF group received two i.p. doses of 2 mg kg^{-1} MTX, 50 mg kg^{-1} CYP and 50 mg kg^{-1} 5-FU over 2 weeks (once per week). The MET group received MET daily in their drinking water at a concentration of 2.5 mg mL^{-1}. The CMF+MET group received two i.p. doses of CMF (2 mg kg^{-1} MTX, 50 mg kg^{-1} CYP and 50 mg kg^{-1} 5-FU) and MET daily in their drinking water (2.5 mg mL^{-1}) over 2 weeks. The animals were observed daily for mortality and their body weights were measured every 3 days. Two days after the second CMF dose was given, animals in all groups were subjected to behavioural tests.

Y-maze test: The Y-maze test measures an animal's ability to recognize places they have already explored and their ability to explore new places[22]. We used the Y-maze test to assess the ability of the rats to perform hippocampus-dependent tasks and to evaluate their working memory. The Y-maze was made of wood (dimensions: 50×10×18 cm), with three arms placed at a 120° angle to one another. The arms were painted brown

to ensure easy visualization. The apparatus was placed on the floor. The light was provided from above to ensure equal light distribution. In the training session, the animals were allowed to freely explore two arms for 15 min. During the test session (duration: 5 min), the animals were allowed to explore the entire maze, including the novel arm. The time between the two sessions was 3 hrs. The test sessions were video-recorded to determine the time spent in each arm and the number of entries into each arm (note, an animal was considered to have entered an arm if half of its body entered).

Novel Object Recognition (NOR) test: We used the NOR test to evaluate memory function[23]. The test apparatus was composed of a wooden box (dimensions: 40×40×40 cm) with an open top. The familiarization objects were two white teacups and the novel object was a black box of size equal to that of the teacups. In the training session, rats were allowed to explore the two teacups for 10 min before being returned to their cages. In the second session (3 hrs later; duration: 5 min), one of the teacups was replaced with the novel object and the time spent exploring the novel object was recorded using a video camera and a stopwatch[24].

Elevated-Plus Maze (EPM) test: The EPM test is used to measure anxiety, learning and memory processes[25]. The wooden apparatus in this study consisted of two opposing arms: the open arm (50×10 cm) and the closed arm (50×10 cm). The height of the sidewalls of the closed arm was 30 cm. The central platform between the arms measured 10 cm^2. The maze was placed 50 cm above the floor. In the training session, the rat was placed at the end of the open arm, facing the central platform and allowed to explore the apparatus for 10 min. Three hours later, the rat was placed in the same spot as in the training session and the transfer latency time (i.e., the time it took the rat to move from the open arm into the closed arm) and the total time spent in the closed arm were recorded using a video camera[26].

Blood glucose test: On the last day of the experimental period, the tail vein of each rat was injured with a clean sterile needle to obtain optimum-quality blood. An Accu-Chek glucometer with strips was used to test the blood glucose levels of rats in all groups. The equipment was used following the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA): Brains from 12-13 week old control, MET, CMF and MET+CMF rats were collected after Co_2 euthanization of animals and lysed with lysis buffer. The samples were sonicated (Q-Sonica homogenizer, 30 Hz pulses for 20 s) followed by centrifugation at 12,000× g for 10 min. The supernatant was collected, aliquoted into 200 µL vials and stored at −80°C. Protein in the samples was quantified by BCA assay (Pierce). Samples were tested using an ELISA kit containing an interleukin-6 (IL-6) antibody as described in the manufacturer's protocol (MyBioSource, USA). Measurements were made at 450 nm using an ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc.).

Statistical analysis: All results are presented as the mean±standard error of the mean (SEM) and were analyzed using Graphpad Prism 5 software. The survival rate, body weight, Y-maze, NOR, EPM, blood glucose data and ELISA for each group were analyzed by one-way analysis of variance, followed by Dunnett analysis. A p-value <0.05 was considered statistically significant.

RESULTS

Co-administration of MET mitigated CMF toxicity: Treatment with CMF or CMF+MET decreased the survival rate of rats compared with the control and MET-only groups. However, the toxic effect was greater in the CMF-only group than in the CMF+MET group. The toxic effect of CMF was notable 5 days after the initial treatment, with 30% of animals dying within this period. This increased to 50% by day 11. In the CMF+MET group, only 10% of animals died by day 6, although this increased to 50% by day 11 (Fig. 1). No rats died in the control or MET-only groups within the study period.

Co-administration of MET with CMF reduced body weight: The body weights of rats in the CMF and CMF+MET groups were significantly reduced compared with those in the MET-only and control groups ($p<0.001$). The body weights of rats in the MET-only and control groups both increased during the study period, with MET-only treatment resulting in a significantly increased body weight compared with the control group (Fig. 2a-b).

Effects of CMF and MET-only on Y-maze performance: Rats treated with CMF and CMF+MET showed significantly fewer entries into the novel arm in the Y-maze test compared with MET-treatment-only and control rats (Fig. 3a). MET-only treatment reduced the number of entries compared with control rats, but this difference was not significant (Fig. 3a). Rats in the CMF and CMF+MET groups spent less time in the novel arm, but there were no statistically significant differences among the four groups (Fig. 3b).

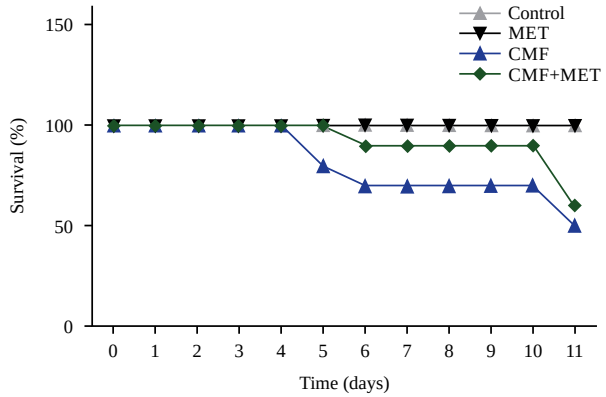

Fig. 1: Effects of CMF (cyclophosphamide [CYP], methotrexate [MTX] and 5-fluorouracil [5-FU]) and metformin (MET) treatment on survival of rats

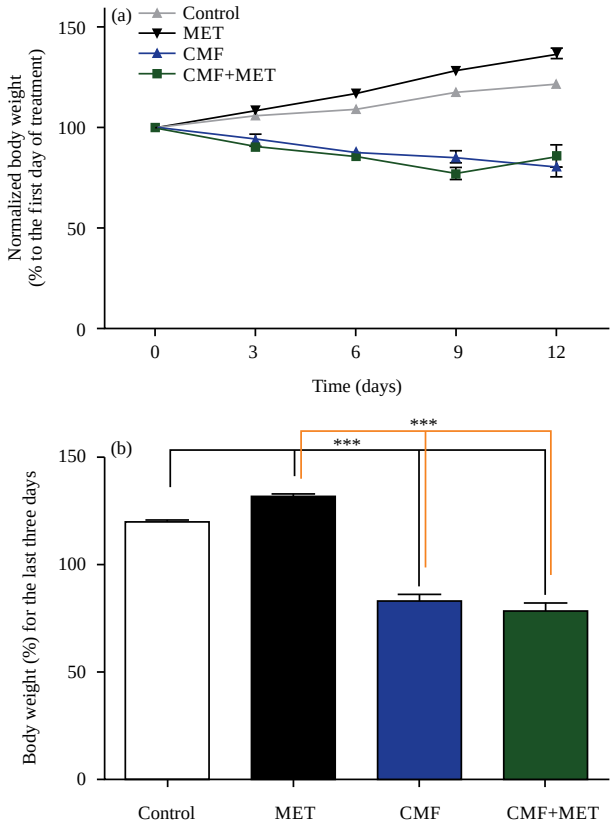

Fig. 2(a-b): Effects of CMF and MET treatment on rat body weight

(a) Normalized body weight over the 2-week study period and (b) Average body weight in the last 3 days of treatment shown as a percentage of the average starting weight (***p<0.01)

Effects of CMF and MET on NOR test performance: CMF and CMF+MET treatment did not significantly affect the performance during the NOR test compared with control animals (Fig. 4).

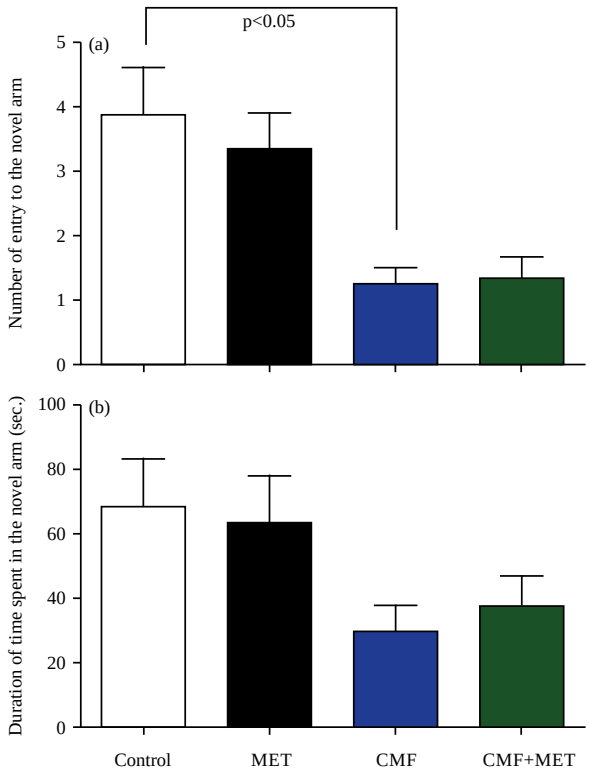

Fig. 3(a-b): Effects of CMF and MET treatment on the performance of rats in the Y-maze test

(a) Number of entries into the novel arm (*p<0.05) and (b) Total time spent in the novel arm

Effects of CMF and MET on EPM test performance: The transfer latency time in the EPM test following CMF+MET treatment was significantly increased compared with that for rats in the control, MET and CMF groups. The transfer latency times in the MET- and CMF-treated groups were not significantly different from those in the control group. This suggests that memory was impaired by treatment with CMF+MET (Fig. 5a). There were no significant differences in the total time spent in the closed arm for the MET, CMF and CMF+MET groups versus the control; however, MET-only treatment significantly reduced the time spent in the closed arm compared with CMF+MET treatment (Fig. 5b).

Effects of CMF and MET on blood glucose levels: Glucose levels in blood were measured 1 day after the completion of treatment. MET- and CMF-treated rats did not show significant changes in their blood glucose levels compared with control animals (Fig. 6). However, there was a significant decrease in the blood glucose level of CMF+MET-treated rats compared with controls.

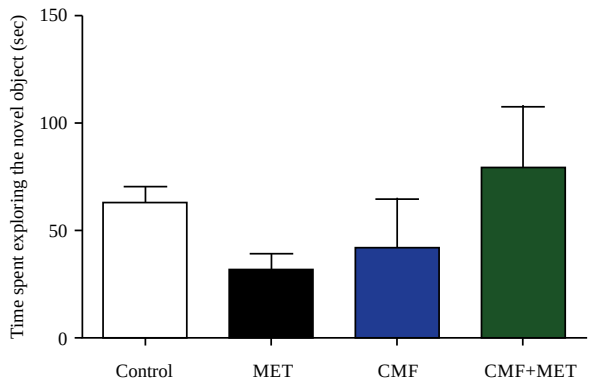

Fig. 4: Effects of CMF and MET treatment on the performance of rats in the novel object recognition test

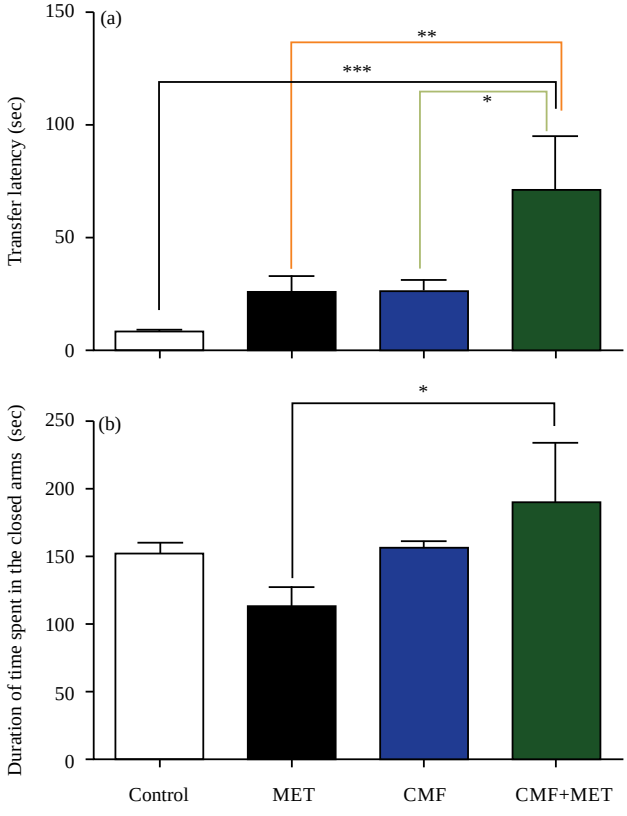

Fig. 5(a-b): Effects of CMF and MET treatment on the performance of rats in the elevated plus-maze test

(a) Transfer latency time (*p<0.05, **p<0.01, ***p<0.001) and (b) Time spent in the closed arm (*p<0.05)

IL-6 levels in rat brain: Rats treated with CMF+MET showed increased IL-6 expression in the brain, significantly so compared with the MET- and CMF-treatment groups (Fig. 7).

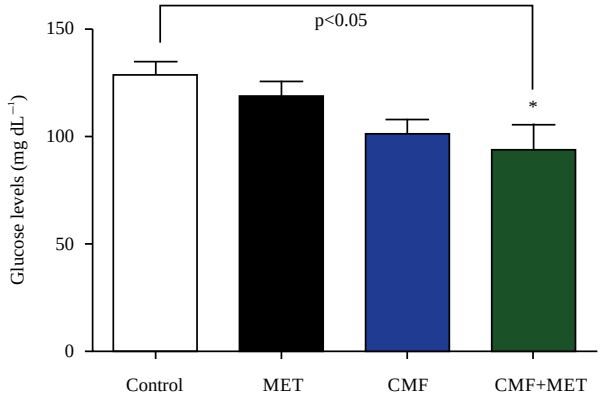

Fig. 6: Effects of CMF and MET treatment on blood glucose levels of experimental rats (*p<0.05)

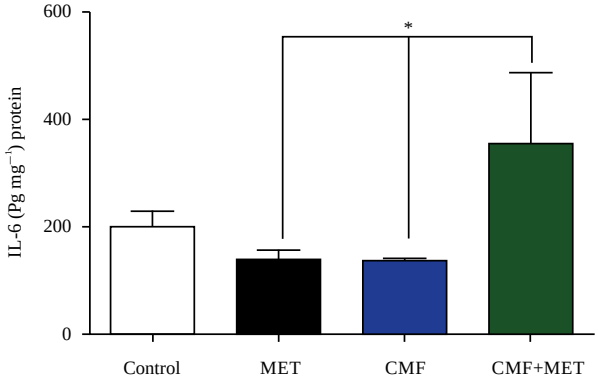

Fig. 7: Effect of MET and CMF treatment on interleukin-6 expression in rat brain

DISCUSSION

In the present study, we evaluated the protective effect of the antidiabetic agent MET against toxicity and memory impairment induced by the CMF chemotherapeutic protocol [CYP (50 mg kg^{-1}), MTX (2 mg kg^{-1}) and 5-FU (50 mg kg^{-1})] in an albino rat model.

MET has been reported to improve the quality of life of diabetes patients by regulating blood glucose levels. It also reduces the risk of occurrence of Alzheimer's disease[27]. Furthermore, previous studies have revealed that MET potentially reduces the toxic effects of chemotherapy and therefore increases the survival rate, reduces cardiotoxicity and improves cognitive dysfunction resulting from chemotherapy[10,12,28]. In the present study, it was hypothesized that MET could improve cognitive impairment caused by CMF treatment and improve the survival rate of treated animals.

Several lines of evidence have revealed that MET enhances the effect of chemotherapy against cancer growth[29]. MET activates AMPK, which both activates and inhibits other proteins. For instance, AMPK phosphorylates and inhibits the mTOR protein, which is important in cell proliferation[30]. CYP, which is used in the CMF chemotherapeutic protocol, also inhibits mTOR[31]. Several studies have shown that the combination of chemotherapy and MET treatment can increase the effect of chemotherapy on the cancer cells, as well as the toxic effect of treatment on normal cells[32,33]. In the present study, a lower CMF dose was used. This allowed elucidation of the mitigating effect of MET on CMF toxicity. CMF+MET-treated animals showed increased survival in the initial days of treatment compared with CMF-treated animals (Fig. 1). However, the reduction in body weight associated with CMF treatment was not mitigated by co-administration of MET, with rats in both the CMF and CMF+MET groups showing a reduction in body weight compared with controls over the 2-week study period (Fig. 2).

To evaluate working and spatial memory, the Y-maze test was used in this study and the results revealed that the memory was impaired by CMF treatment, with rats in the CMF-treatment-only group showing a significantly decreased number of entries into the novel arm compared with control animals (Fig. 3). Animals treated with CMF+MET also showed a decreased number of entries into the novel arm compared with controls, although this difference was not statistically significant. Furthermore, CMF and CMF+MET-treated rats spent less time in the novel arm compared with control animals, although these changes were, again, not statistically significant. These data suggest that cognitive function is affected by CMF treatment.

The EPM task was used as another tool to study the behaviour and cognitive function of rats in this study. The test revealed that a longer transfer latency time was associated with CMF+MET treatment compared with all other groups (Fig. 5a). However, the total time spent in the closed arm was reduced in the MET-treated group compared with the CMF+MET-treated group (Fig. 5b). These results indicate that the longer transfer latency time following CMF+MET treatment may be due to memory issues and not as a result of lethargy resulting from the treatment.

The effect of chemotherapy on blood glucose levels is controversial. Some studies have reported that some of the chemotherapeutic agents potentially increase glucose levels, whereas other studies indicated that other chemotherapeutic agents did not affect glucose levels[34-36]. The acute effects of doxorubicin, CYP and 5-FU did not alter glucose levels[33]. In the present study, the blood glucose levels of control, MET-only, CMF-only and CMF+MET-treated animals were evaluated. The levels in CMF-only or MET-only treated animals did not differ compared with controls. However, in the CMF+MET treatment group, the blood glucose level was significantly decreased compared with the control group. MET is known to reduce and regulate glucose to normal levels[37,38]. Our data indicate that the combination of CMF and MET can decrease glucose levels below normal and this could be a result of a reduction in the quantity of food intake. The result of Fig. 2 shows a highly significant decrease in rat body weight in CMF+MET treated animals compared with controls. It has been reported previously that cancer patients lose appetite and appreciation of food after chemotherapy[39].

Taken together, these data suggest that CMF treatment can somewhat impair cognitive function by affecting the hippocampus. Furthermore, our findings from the EPM task showed that co-administration of MET with CMF may enhance this cognitive dysfunction. Overall, the behavioural assessments demonstrated that MET treatment failed to improve the cognitive deficits caused by CMF treatment.

IL-6 is a pro-inflammatory marker that is expressed in the central nervous system. IL-6 is known to affect synaptic plasticity and neuronal function and thus learning and memory processes[40]. Increased levels of IL-6 are associated with memory deficits[41]. In the current study, IL-6 expression was increased in the MET+CMF treatment group, whereas it was not altered in the MET-only and CMF-only groups, compared with controls. Therefore, the combination of MET and CMF might cause cognitive impairment by increasing the level of IL-6 expression.

To the best of the author's knowledge, this is the first study that assesses the effect of the co-administration of MET with CMF on cognitive dysfunction using a rat model. An important strength of this study is that the CMF dosage regime used was selected to be clinically relevant to the dose used in cancer patients. An additional strength is that the rats used were of the same strain and age and all the experiments were conducted concurrently among the study groups to avoid the effects of confounding factors. It should also be noted that the rats used in this study were free of cancer and therefore the effects observed can be assumed to be as a result of MET and CMF treatment, rather than cancer itself.

CONCLUSION

MET mitigated the toxic effect of CMF and increased the survival rate in the initial stages of CMF treatment, but it did not fully reverse mortality and did not have a beneficial effect on body weight. MET treatment failed to improve the

cognitive deficits caused by CMF treatment. Indeed, MET combined with CMF could further impair memory function according to the EPM test. CMF+MET-treated rats had significantly increased levels of IL-6 in the brain compared with CMF-treated animals and controls, suggesting increased neuroinflammation.

SIGNIFICANCE STATEMENT

The findings from this study identify that two weeks of treatment of CMF affected the mortality rate, body weight and memory function in rats. This such that the treatment decreases the number of entries to the novel arm, duration of time spent in the novel arm and increase the transfer latency in CMF and MET treated rats. The study revealed that the longer duration of CMF therapy in animals such as rats could modulate cognitive functions. These effects of drugs that occurs during the normal course of chemotherapy treatment go noticed and well documented; however, the mechanism of these changes are not well understood. Considering the therapeutic applications of anticancer such as CMF protocol, the findings of this study could provide a platform for future research to explore the effect of CMF on memory function and cognitive impairments in both experimental and clinical human subjects.

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