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Research Article
Synergistic Effects of Metformin and Fluorouracil on Cognitive Disability via Inducing Neuroinflammation in Rat Models

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Abstract

Background and Objective: Fluorouracil (FU) is a potent chemotherapeutic agent commonly used in combination with other drugs to effectively combat various sorts of cancer, including breast, prostate, lung and brain cancer. The Metformin (MET) is a medication that has been found to potentially improve cognitive disability connected with diabetes and Alzheimer's disease. This study assessed the potential preventive effects of MET on cognitive disability induced by FU. **Materials and Methods:** Male rats in the FU and FU+MET groups received intraperitoneal injections of FU (100 mg/kg) the control group received saline via the same route. Rats in the MET and FU+MET groups were then offered drinking water containing MET (2.5 mg/mL). The cognitive ability of rats was then evaluated in behavioral tests (Y-maze, novel object recognition, elevated plus maze), while inflammation in the hippocampus was assessed by analysis of the levels of TNF-α, IL-6 and IL-1β in enzyme-linked immunosorbent assays. **Results:** The findings indicated an increase in mortality and a reduction in body weight among rats treated with FU compared to the control. The FU treatment also impair memory function by decreasing the exploration of the novel arm in Y-maze and novel object in NOR tests and increasing the transfer latency in EPM. Compared with the control group, increased TNF-α, IL-6 and IL-1β levels were detected in the hippocampus of rats in the FU and FU+MET-treated groups. **Conclusion:** The findings indicate that FU-induced cognitive disability was caused by increased neuronal inflammation and that this effect was further enhanced by MET.

Key words: Fluorouracil, metformin, cognitive disability, interleukin-1, interleukin-6, TNF-α

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Competing Interest: The author has declared that no competing interest exists.

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

INTRODUCTION

Chemotherapy can cause a range of adverse side effects, including fatigue, pain, hepatotoxicity and memory loss[1,2]. Studies have indicated that cognitive disability may occur following chemotherapy both in experimental and clinical settings[3]. Long-term cognitive disability following chemotherapy is a well-documented phenomenon known as "chemobrain" that poses a significant challenge for individuals who have survived cancer, with rates varying from 17 to 75%[4-6].

Certain substances are known to have the capability to cross the blood-brain barrier (BBB) and it was previously believed that only chemotherapeutic drugs with this ability could potentially cause memory dysfunction[7,8]. However, it has been discovered that the cognitive effects are caused regardless of the ability to cross the BBB[9]. Fluorouracil (FU) is a widely used anticancer drug that has proven effective in treating various sorts of cancer, including brain, lung and breast cancers[10]. The FU has been reported to cause memory dysfunction[11] via a mechanism that involved decreased dopamine release, increased oxidative stress and neuronal degeneration[12,13].

There is increasing evidence supporting the positive effects of metformin (MET), a widely prescribed medication for diabetes, on various diseases beyond diabetes[14]. For example, prolonged use of MET has been linked to its ability to combat cancer and increase lifespan[15]. Nevertheless, studies examining the impact of MET on cognitive function have thus far yielded controversial results. Studies have shown positive effects of MET memory in rats, while others have found contrasting results when the drug was administered to healthy rodents[16]. The MET has also been shown to lower the probability of acquiring neurodegenerative illnesses[17]. However, some diabetic patients have reported potential negative effects on cognitive performance when using MET for an extended duration[18].

There are multiple indications that MET could potentially be beneficial in preventing chemobrain[19] by inhibiting inflammation and decreasing oxidative stress[20]. The MET co-administration has been shown to alleviate the negative effects of chemotherapy on the kidneys and liver and improve memory problems caused by cisplatin and cyclophosphamide treatments[21]. In addition, a previous study demonstrated that MET administered alongside cyclophosphamide effectively rescued behavioral disability in an animal model of cyclophosphamide-induced chemobrain[19]. Despite such reports of the positive effects of MET preventing chemobrain, contradictory evidence also exists. For example, MET did not alleviate the memory disability caused by doxorubicin therapy[16]. Furthermore, recent studies have shown that the combination of MET and FU can lead to intestinal injury by suppressing inflammation and oxidative stress[22]. On the other hand, MET enhances the effectiveness of fluorouracil by disrupting ATP production and DNA repair in breast and colon cancer cells[23,24].

Therefore, the current study assessed the impact of FU on cognitive function and the potential of combining MET with FU to mitigate any cognitive deficits caused by FU. Furthermore, changes in the concentrations of TNF-α, IL-6 and IL-1 are measured to evaluate the role of neuroinflammation in the underlying mechanism.

MATERIALS AND METHODS

Study duration: The study was conducted from November, 2023 to December, 2023 at a Pharmacology Laboratory in the College of Pharmacy at Qassim University, Saudi Arabia.

Animal handling: Forty male albino rats (aged 12 weeks, 200-250 g) obtained from the animal house in the College of Pharmacy, Qassim University were housed individually in a highly regulated sterile environment under a 12 hrs light and 12 hrs dark cycle (lights on at 6:30 am) at the Pharmacy College of Qassim University, Kingdom of Saudi Arabia. The rats had free access to the water and were fed a standard chow diet. The animals were monitored daily to detect any potential alterations in mortality rate and body weight. Behavioral trials were carried out during the light period.

Ethical consideration: The study was approved by the Standard Animal Ethics Guidelines established by the Institutional Committee on Animal Protection and Use of the Qassim University's Deanship for Scientific Research (approval number: 22-16-02) in accordance with international norms followed in this study.

Drug administration: Rats received saline and FU (100 mg/kg) delivered by intraperitoneal injection once every third day (total 3 doses). The animals were injected with saline or FU by intraperitoneal route of administration. In this, the needle was inserted into lower right quadrant of the abdomen towards the head at an angle 30-40° horizontal. The plunger of the syringe was slightly pulled back before injection. The appearance of a small air bubble confirmed the intraperitoneal route[25]. The MET was added to the drinking water at a concentration of 2.5 mg/mL. Behavioral tests were performed one day after the final dose was administered.

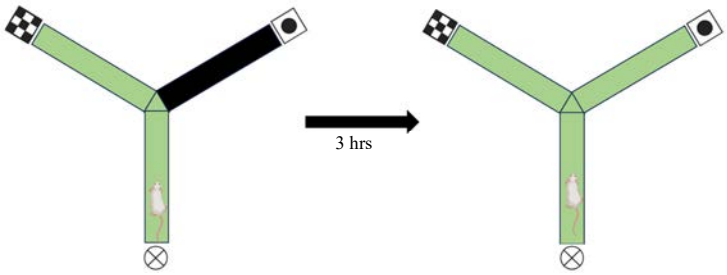

Fig. 1: Illustration of the Y-maze utilized in the study of associative learning and memory, with observed cues located at the ends of the arms

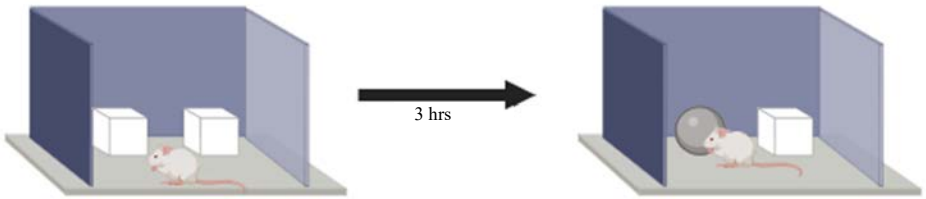

Fig. 2: Schematic diagram of novel object recognition task apparatus

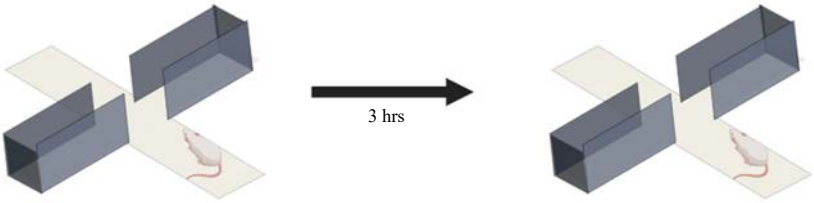

Fig. 3: Elevated plus maze apparatus used to assess anxiety and memory

Y-maze test: Increasing the amount of time spent or the number of entries made into the novel arm is believed to enhance the ability to uncover new information. The Y-maze was constructed from wood, with dimensions of 50×10×15 cm (Fig. 1). To improve visibility, the Y-maze arms were painted brown and angled 120°, with a lamp above each arm to distribute light evenly across the equipment. Each experiment was filmed. The rats were tested in both exploratory and experimental sessions during the Y-maze test, which displayed circles, triangles and crosses at the end of each arm. In the initial exploration session (15 min), animals explored both Y-maze arms to the left or right of the starting arm. One arm was blocked during exploration to see if the rat explored the unfamiliar arm and both were opened during the experiment. After 3 hrs, rats were placed in the same arm as in the first exploration session for 3 min for the second exploration session, allowing them to wander the maze freely. More than half of a rat's body was inside its arm[16].

Novel object recognition (NOR) test: Figure 2 depicts the schematic of the NOR task apparatus used to test learning and memory. Its wooden box is 40×40×40 cm. Rats explored two novel teacups for 15 min before returning to their cages. After 3 hrs, one teacup was replaced with a black box similar to the teacup size and the rats were returned to the experimental setup. The 3 min exploration of the novel object was recorded and analyzed[16].

Elevated plus maze (EPM) tests: Figure 3 shows the EPM used to assess anxiety and memory. The apparatus is wooden with open arms (50 cm lengths×10 cm width) and closed arms (50 cm lengths×10 cm width×30 cm sidewalls) forming a cross with a 10 cm^2 medial axis. The maze was 50 cm high. The rat was permitted 10 min to investigate the device while sitting at the end of an open arm facing away from the medial platform. After 3 hrs, the rat was reinstalled in the apparatus at the same spot investigated in the exploration session and transfer delay time and arm time were measured[26].

Enzyme-Linked Immunosorbent Assays (ELISA): On day 10 after the last dose of medication, the rats were euthanized by CO_2 asphyxiation. Euthanasia in experimental animals was done as per the procedure described in the literature. The euthanasia chamber was filled with 50% CO_2 for 5 min[27]. Brains were removed from all groups (saline control, FU, MET and FU+MET) and the hippocampi Campus region was isolated for processing with Neuronal Lysing Buffer (N-PER) using a Q-Sonica homogenizer. After centrifugation at 12,000 g for 10 min, the supernatant was collected and stored at -80°C in 200 µL tubes for future use. The protein levels of the samples were determined utilizing the bicinchoninic acid test (Pierce, Waltham, Massachusetts, USA). The TNF-α, IL-6 and IL-1β concentrations in the samples were determined using Enzyme-Linked Immunosorbent Assay (ELISA) kits earned from (MyBioSource, San Diego, California, USA) according to the manufacturer's instructions[28]. The concentrations of the inflammatory mediators were detected utilized rat ELISA kits for TNF-α1 (cat. no. RK00029, ABclonal Technology), IL-6 (cat. no. RK00020, ABclonal Technology) and IL-1β (cat. no. RK00009, ABclonal Technology, Woburn, Massachusetts, USA).

Statistical analysis: Data were presented as the Mean±Standard Error of the mean (SEM). All statistical analysis was performed using GraphPad Prism 10. Differences between groups were evaluated by One-way Analysis of Variance (ANOVA) followed by Tukey's test. A $p<0.05$ was considered to indicate statistical significance.

RESULTS

FU combined with MET decreases the survival rate: As shown in Fig. 4, there was a decrease in the survival rate in the FU and FU+MET groups compared to that in the saline control group. However, the survival rate in the FU+MET was lower than that in the FU group (80 vs 90%, respectively). There were no fatalities in the MET group. The FU alone resulted in a 10% mortality rate, while the combination of FU and MET led to a 20% mortality rate. In contrast, all the saline and MET groups survived throughout the study.

FU combined with MET reduces body weight: As shown in Fig. 5(a-b), it is observed a reduction in the body weight of the FU and FU+MET groups compared with that in the saline control and MET groups.

FU combined with MET impairs memory on Y-maze task performance: Figure 6 provides an overview of the performance of rats in the Y-maze task conducted to evaluate spatial memory. Compared with the saline control group, it is observed a decrease in the number of entries into the novel arm by rats in the FU, MET and FU+MET groups, although this effect was significant only in the FU+MET group ($p<0.05$) (Fig. 6a). Furthermore, rats in the FU alone and combination with MET reduced the tendency of rats to explore unfamiliar areas as compared with rats in the saline control group ($p<0.01$ and $p<0.001$, respectively) (Fig. 6b).

Effects of FU and MET treatments on NOR task performance: An overview of the performance of rats in the NOR task conducted to assess learning and memory function is shown in Figure. Compared to the saline control group, there was notable reduction in the time spent exploring the novel object in the FU and FU+MET groups, while this parameter remained unchanged in the MET group (Fig. 7). These findings suggest that the treatments with FU and FU+MET have an impact on memory function in rats.

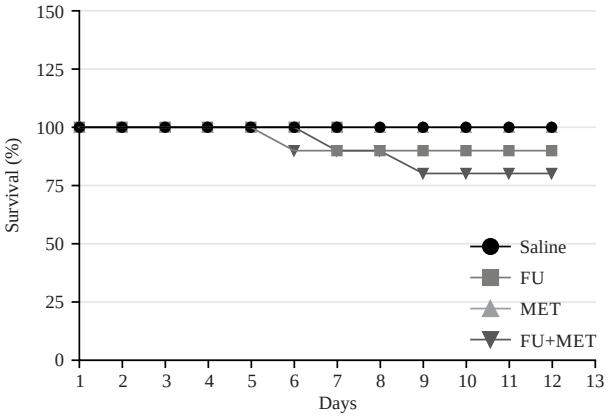

Fig. 4: Influence of FU and MET alone and in combination on survival rate

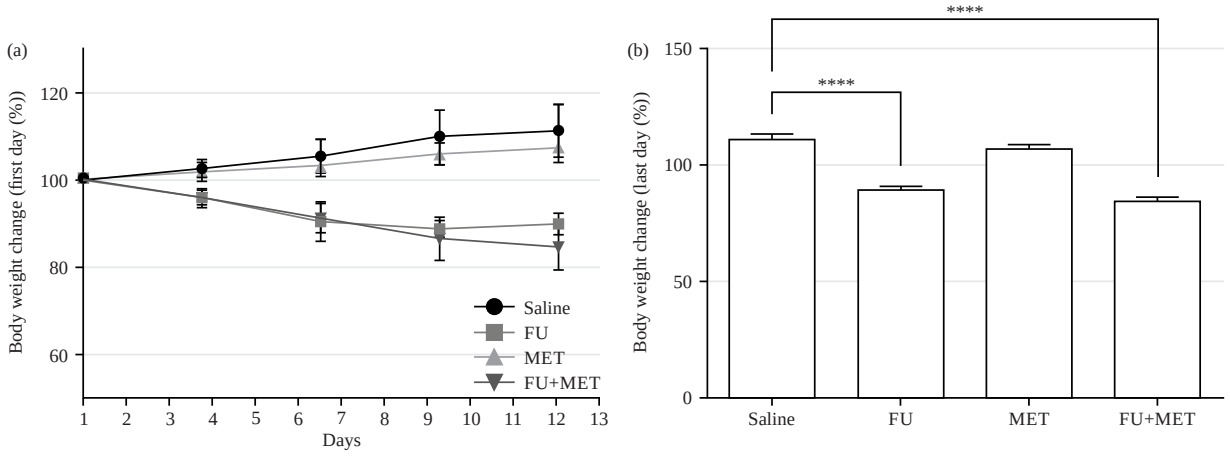

Fig. 5(a-b): Impact of FU and MET and combination on the body weight, (a) Study measured modifications in body weight from the start to the experiment's conclusion, which showed a significant decrease in FU alone and combined FU and MET related to the saline and MET alone and (b) Percentage change observed on the final day of the study revealing a significant reduction in FU and MET related to the saline and MET alone

****p<0.0001

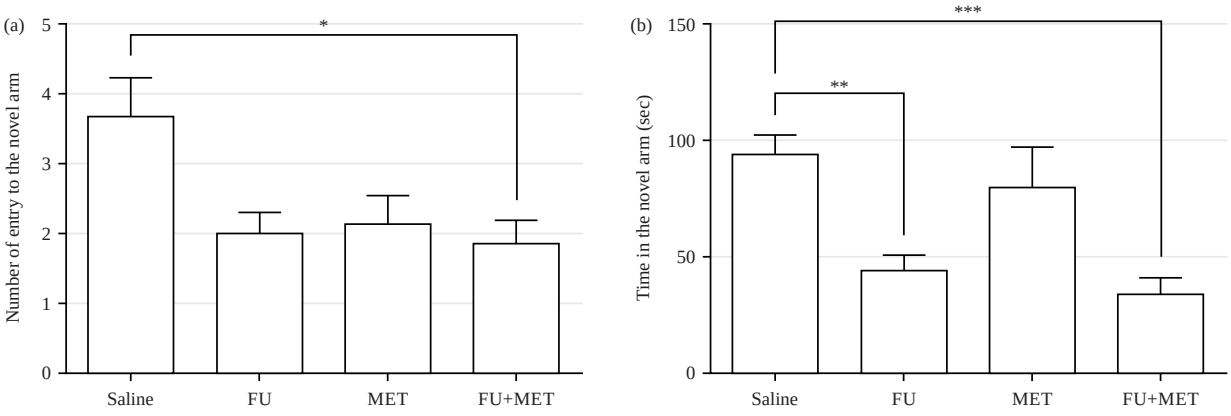

Fig. 6(a-b): Influence of FU and MET and combination on Y-maze behavior task of rats, (a) Quantity of times they entered the novel arm and (b) Amount of time they spent on the novel arm

(a) Revealed reduction in MET, FU and combined MET with FU whereas only combined MET with FU was statistically significant, (b) Showed a reduction in time spent in the novel arm in FU and combined MET with FU related to saline group, *p<0.05, **p<0.01 and ***p<0.001 as equated to the saline

Effect of FU and MET treatments on EPM task performance: An overview of the performance of rats in the EPM task conducted to assess memory function was shown in Fig. 8. Compared to the saline control group, the transfer latencies of rats in the EPM task were higher in the FU, MET and FU+MET groups, although the difference was statistically significant only in the FU+MET group ($p<0.01$). These findings suggest that the combination of FU and MET had a more detrimental effect on memory in rats compared to FU alone.

Effect of FU and MET on IL-6, IL-1β and TNF-α levels in the hippocampus: To evaluate the potential of MET to alleviate FU-induced inflammation, it is measured the concentrations of inflammatory mediators (IL-6, IL-1β and TNF-α) in rat hippocampal tissue by ELISA. Compared to the saline control group, higher levels of TNF-α, IL-6 and IL-1β were detected in the hippocampus of rats in the FU group (Fig. 9a-c). However, the combination of MET with FU did not reverse these effects to the same extent as the saline group.

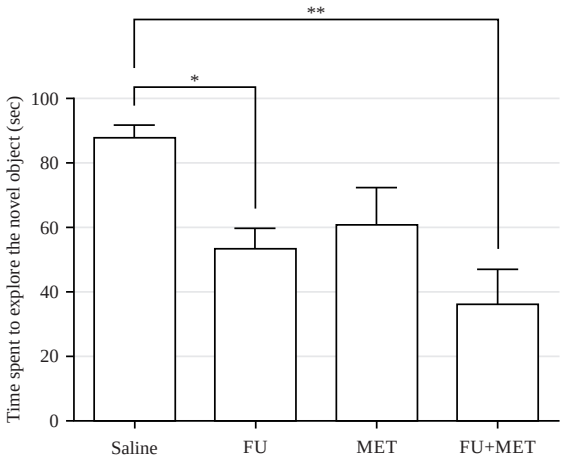

Fig. 7: Influence of FU and MET and combination on NOR behavior tasks of rats

Time spent exploring the novel object in the FU and MET plus FU groups was significantly reduced related to the saline group indicating inability of rats recognizing the new object, data was the Mean±Standard error of the mean when *p<0.05 and **p<0.01 as equated to the saline

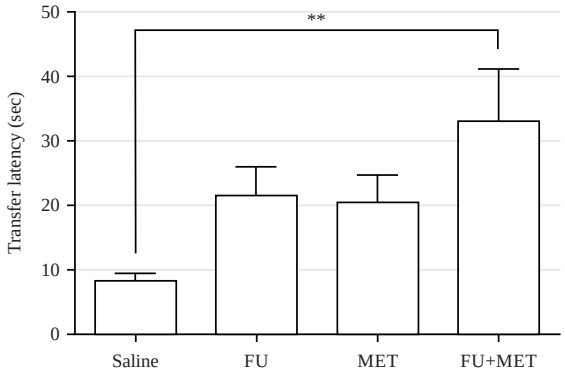

Fig. 8: Influence of FU and MET and combination on transfer latency in the EPM task

Statistically significant representation of the data was the Mean±Standard error of the mean and **p<0.01 as equated to the saline

DISCUSSION

The current investigation utilized FU-treated experimental rats to simulate chemobrain and examine the impact of MET on FU-induced toxicity, focusing on its influence on neuroinflammation as well as learning and memory behaviors. Multiple lines of evidence have indicated a correlation between chemotherapy and cognitive disability[3,29,30]. While the precise cause of this phenomenon remains uncertain, studies suggest that it may be linked to neuroinflammation, changes in synaptic protein expression and decreased neurogenesis[31,32]. As a potent chemotherapist agent used in chemotherapy regimens, FU is classified as an antimetabolite[33]. The FU inhibits DNA synthesis, leading to cancer cell death and tumor destruction; however, it also affects brain neurons, which can lead to cognitive disability[34,35]. Although the mechanism remains to be fully elucidated, neuroinflammation has been implicated in the process by which chemotherapy can induce cognitive impairment[3]. The MET is an antidiabetic drug that has been found to help reduce the toxicities associated with chemotherapy such as cisplatin and cyclophosphamide[19,21]. In the current study investigates the ability of FU to cause cognitive impairment in rats and explored the potential of MET to reduce the neuronal toxicities and inflammation caused by FU. By assessing the performance of rats in behavioral tasks such as the Y-maze, NOR and EPM and measuring levels of inflammation markers in the hippocampus, the study demonstrates a notable disturbance in hippocampal plasticity and memory in animals treated with FU, which was not alleviated by co-treatment with MET.

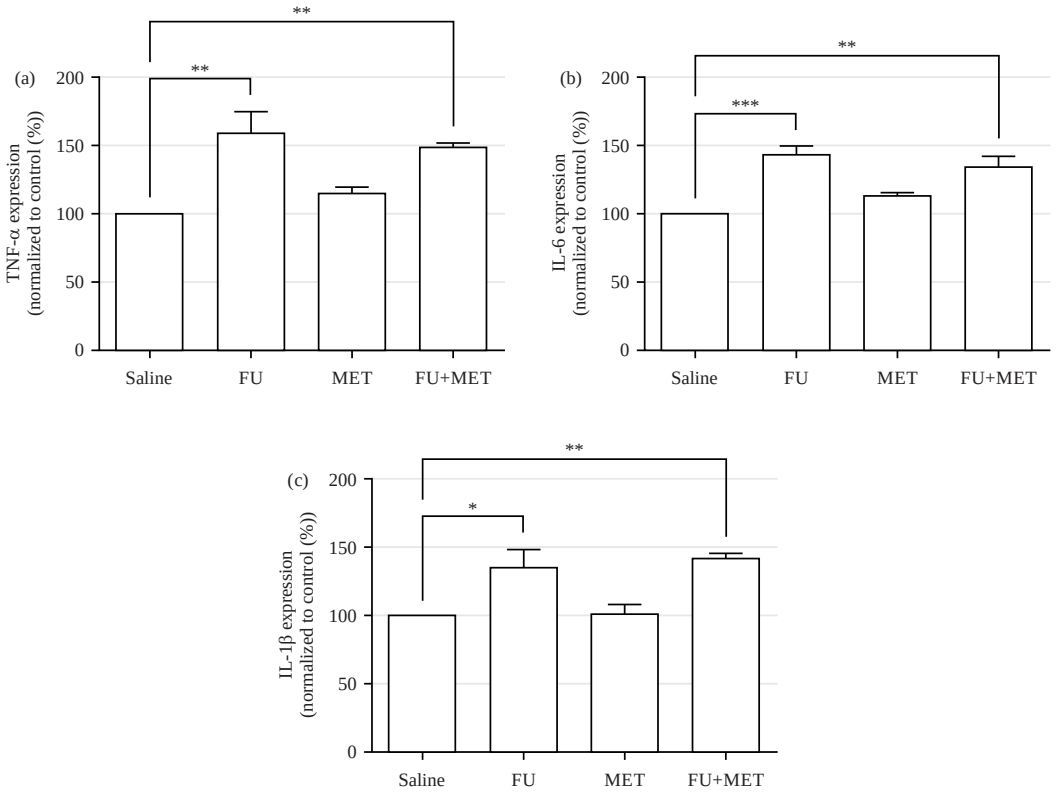

Fig. 9(a-c): Influence of FU, MET and combination on hippocampal, (a) TNF-α, (b) IL-6 and (c) IL-1β concentrations in rats

A statistically significant representation of the data was the Mean±Standard error of the mean when $p<0.05$ as equated to the saline, *$p<0.05$, **$p<0.01$ and ***$p<0.001$

Evaluation of survival rate and body weight, which are indicators of toxic effects[36], is crucial in studies that assess the effectiveness of medication or drug interactions[36,37]. In this study, it is observed a survival rate of 100% in the saline control and MET groups, while survival in the FU group was reduced to 90% and in the FU+MET group, the rate was further reduced to 80%. In addition, compared with the start of the study, the body weights of rats in the saline control and MET groups had increased by 11 and 8%, respectively, at the end of the experimental period. In contrast, the body weights of rats in the FU and FU+MET groups had decreased to 10 and 15% of their initial weights, respectively. Overall, these observations indicate that, although FU causes toxicity that decreases the survival rate and body weight of rats, the combination of FU and MET had a more significant impact than FU alone.

Recent research has shown that chemotherapy, which included cisplatin, doxorubicin and cyclophosphamide, had a negative impact on cognitive performance when hippocampus-dependent behavior tests were used. Cognitive function improved significantly when MET was combined with cyclophosphamide and cisplatin, whereas no improvement was observed when doxorubicin was administered. The present study has explored the changes in the cognitive function of rats after administering FU and MET, alone and in combination by assessing their performance in three behavioral tasks (Y-maze, NOR and EPM). In the Y-maze task, rats treated with FU showed a decline in the number of entries to the new arm and spent less time there than those in the saline control group. Furthermore, the significant reductions in the number of entries and time spent in the novel arm by the rats treated with the combination of MET and FU indicated a negative impact on memory function as compared to the saline group. In the NOR task, FU and the combination of MET with FU led to a notable decrease in the time spent exploring the novel object compared with the familiar object, indicating a decline in the ability to differentiate between the two objects. In addition, the outcomes of the EPM study revealed a higher transfer latency in all the groups that received treatment (FU, MET and a combination of MET with FU), although only the MET+FU group showed a statistically significant increase. Overall, the behavioral findings indicate

that FU can drive cognitive disability and that the combination of MET and FU may exacerbate this negative impact on cognition.

Several studies have established a connection between chemotherapy and neurotoxicity as well as cognitive loss due to the influence that chemotherapies, such as doxorubicin and cyclophosphamide, which elevate the release of inflammatory proteins[38-40]. Additionally, a sustained rise in the expression of inflammatory proteins in the brain is linked to the occurrence of neuropathic pain associated with chemotherapy[41]. In addition to the effects of chemotherapy, increased production of inflammatory proteins, such as TNF-α, IL-6 and IL-1β, can be a characteristic of the tumor itself[4,42,43]. These inflammatory proteins can spread throughout the entire body through the bloodstream[44] and can also enter the brain by crossing the BBB, resulting in the loss of the barrier's integrity and activation of inflammation in the central nervous system as a reaction to peripheral inflammation[45]. In addition, FU can penetrate the brain and may result in neurotoxicity by causing neuronal damage[46], which can lead to neuroinflammation through elevation of the concentrations of proinflammatory proteins[38]. In accordance with previous research, the findings of the study revealed increased levels of TNF-α, IL-6 and IL-1β levels in the hippocampus of rats after FU treatment as compared with the saline controls[38]. Moreover, this increase in neuroinflammatory proteins in rats treated with FU has been linked as a cause of the cognitive decline[38]. It has been reported that MET possesses properties that can potentially reduce the toxic effects of doxorubicin by acting as an anti-inflammatory and antioxidant agent[47,48]. Furthermore, studies conducted on experimental rodents and humans have shown that MET can effectively prevent the formation of β-amyloid and reduce the production of TNF-α[49-51], which can lead to an improvement in memory function, especially in individuals with diabetes[50]. In contrast to these findings, the combination of MET and FU in the present study led to a synergistic effect, which had a negative impact on cognitive function and increased neuroinflammation. The observed impact of MET may be attributed to its capacity to disrupt brain bioenergetics and impede the activity of crucial proteins involved in cognitive processes, such as the mammalian target of rapamycin (mTOR) and Extracellular Signal-Regulated Kinase (ERK)[18,52].

The chemotherapy-induced cognitive dysfunctions and neuronal toxicity are more challenging during and after the treatment of cancer patients[53]. Therapeutically, FU is frequently used in the treatment of various types of cancers, including colorectal, breast, gastric and pancreatic cancers[54]. Also, it is widely reported with induction of neurotoxicity; however, the toxicity profiles discourage its usage[55]. The current study has covered potential neuroinflammation mechanisms by elevation of TNF-α, IL-6 and IL-1β related to FU neurotoxicity. Also, the targeted drug MET is believed to provide some positive therapeutic uses against FU-induced neuronal-related toxicities[22]. Nevertheless, the results of this study revealed a collaborative intervention between MET and FU that increased neuroinflammation. Practitioners need to take these toxicities into account when administering treatment. Furthermore, this opens up opportunities for additional research focused on investigating and gaining a deeper understanding of the underlying toxicities.

This study exhibits both advantages and limitations. It is the first study to explore the influence of FU combined with MET on cognitive function. Additionally, it examined the potential link between MET and FU treatments and neuroinflammation. To minimize any possible bias, all the animals used in this study were matched for age and strain and each trial was conducted concurrently. In addition, all animals were cancer-free to allow a concentrated evaluation of the specific impacts of FU treatment, while reducing any possible interference from malignancy. The treatment involved administering multiple doses, which is similar to the approach used for cancer patients. Furthermore, the examination of the histopathology would provide more convincing evidence of inflammation in the hippocampus; however, such investigations were hindered by a lack of experience in histopathology examinations and the laboratory's limitations for performing this technique.

CONCLUSION

The use of Y-maze, NOR and EPM behavioral tasks, the current study confirms that cognitive impairment is induced by FU and provides evidence that this effect is linked to increased levels of inflammatory proteins TNF-α, IL-6 and IL-1β, suggesting a potential mechanism. In addition, it shows that MET did not reverse this neuroinflammation. Ultimately, this study provides a better understanding of the impact of FU and MET on neuronal dysfunction and offers valuable insights for practitioners and researchers interested in understanding drug interactions for chemotherapy-induced neurotoxicity.

SIGNIFICANCE STATEMENT

This study seeks to assess the potential therapeutic advantages of metformin in mitigating cognitive impairment caused by fluorouracil. According to the research findings, rats that received a combination of metformin and

fluorouracil showed a decline in their behavioral function and an increase in the levels of certain neuroinflammation mediators (TNF-α, IL-6 and IL-1β). According to the results, it seems that metformin is not able to effectively alleviate the neurotoxic effects caused by fluorouracil. This research enables practitioners and researchers to gain a deeper understanding of the toxic effects of combining metformin and fluorouracil. This knowledge can greatly benefit their practice and inspire the development of alternative and innovative strategies.

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