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Effects of CMF and MET on glutamate and dopamine levels in the brain, and their impact on cognitive function

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Abstract. – OBJECTIVE: Chemotherapy can cause cognitive impairment in cancer survivors. CMF, the combination of cyclophosphamide (CYP), methotrexate (MTX), and 5-fluorouracil (5-FU), is employed for the treatment of several types of cancers, such as metastatic breast cancer. Metformin (MET) is an antidiabetic medication used to treat type 2 diabetes that can reportedly alleviate some toxic effects. In the current study, we investigated the ability of MET to alleviate the effects of CMF in neuronal toxicity.

MATERIALS AND METHODS: Rats were treated with two doses of CMF (intraperitoneal injection) and MET (in the daily drinking water). Rats were subjected to fear conditioning memory tests to evaluate memory function following treatment, and brain samples were collected and homogenized using neuronal lysis buffer for assessment of glutamate and dopamine levels by high-performance liquid chromatography (HPLC).

RESULTS: Fear conditioning memory tests revealed a significant reduction in memory function in CMF and CMF+MET groups *vs.* controls, but no significant change in MET groups *vs.* controls was detected. Similarly, CMF and CMF+MET groups revealed a significant increase in glutamate and dopamine levels in the brain of MET, CMF, and MET+CMF groups *vs.* controls based on HPLC results. In addition, although glutamate and dopamine levels were increased, levels varied between groups, with highest levels in the CMF+MET group.

CONCLUSIONS: Our results demonstrate that cognitive impairment in CMF and CMF+MET groups could result from increased glutamate and dopamine levels in the brain, leading to brain toxicity and failure of MET to alleviate the toxic effects of CMF.

Key Words:
CMF, MET, Glutamate, Dopamine, Cognitive function.

Introduction

Chemotherapy can successfully treat various types of cancer[1], and the main mechanism of action is inducing cytotoxicity[2]. However, toxicities associated with chemotherapy can lead to acute and chronic adverse effects, such as cardiotoxicity, nephrotoxicity, and cognitive impairment, known as chemotherapy-induced cognitive impairment, chemobrain, or chemofog[3-5]. Cognitive impairment varies among cancer survivors from moderate to severe, and it affects emotions, behavior, and mental status, which ultimately influences overall quality of life. Unfortunately, therapeutic strategies to reduce neurotoxicity following chemotherapy are limited. Clinical and experimental studies have reported chemotherapy associated with impairment of cognitive function following several chemotherapeutic agents, including cyclophosphamide (CYP), methotrexate (MTX), fluorouracil (5-FU), doxorubicin (DOX), and cisplatin[6-9]. These chemotherapeutic agents were shown to severely impair hippocampus-dependent cognitive function in rodents[10]. These cognitive

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impairments have been attributed to a reduction in neurogenesis, alterations in protein expression and function, and inflammation[8,11,12]. Indeed, we previously showed that acute CMF, a combination of cyclophosphamide (CYP), methotrexate (MTX), and 5-fluorouracil (5-FU), is associated with reduced hippocampal-dependent behavior and increased levels of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and IL-1[9].

In the current study, we evaluated fear conditioning memory, as well as glutamate and dopamine levels in the brain following CMF treatment, and we explored the mechanisms of chemobrain and potential protection of MET against these toxicities.

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS), and it plays distinct roles in normal brain functions and the pathogenesis of various neurological disorders[13]. Glutamate receptors and transporters are key players regulating glutamate release and extracellular glutamate concentrations to maintain dynamic synaptic signaling processes and memory function[14]. Glutamate binds to two major receptor types; ion channels and G protein-coupled receptors (GPCRs). These receptors regulate many physiological functions, including learning and memory processes[15,16]. Briefly, once presynaptic neurons are stimulated, calcium channels are activated, which stimulates Ca^{++} influx into presynaptic terminals, leading to synaptic vesicle localization toward the synaptic membrane, and the release of glutamate. Glutamate binds to both ion channels and GPCRs, which activates several protein cascades and enhances memory formation. Alterations in glutamate levels and/or the expression of glutamate receptors can alter cognitive functions.

Dopamine is a neurotransmitter present in both the CNS and peripheral nervous system[17]. Dopamine has two types of receptors, all members of the GPCR superfamily; D1 and D5 are stimulatory type (Gs), and D2, D3, and D4 are inhibitory type (Gi)[18]. Dopaminergic neurons are mainly located in the substantia nigra of the midbrain, which innervates most other brain regions[19,20]. Dopamine and its receptors play important roles in neuromodulation including motor control, mood, muscle movement, and cognitive function[21,22]. The importance of dopamine on neurotransmission for attention and normal cognitive function has long been known[23], deficiencies in dopaminergic circuits are associated with major cognitive decline, and there is a reduction in dopamine levels in patients suffering from Parkinson's disease and some other disorders, such as attention deficit hyperactivity disorder. Interestingly, these declines in cognitive function can be improved following treatments that increase dopamine levels, or activation of dopaminergic receptors through treatment with levodopa, carbidopa, or apomorphine.

The aims of the present study were to evaluate the effects of CMF and MET treatment on fear conditioning memory and explore the potential mechanisms of cognitive impairment by evaluating glutamate and dopamine levels in the brain.

Materials and Methods

Chemicals

CYP (Endoxan) was obtained from Baxter (Mumbai, Maharashtra, India); MTX (Methotrexate) was obtained from Hospira UK Ltd. (Leeds, UK); 5-FU (Utoral) was obtained from Korea United Pharm Inc. (Seoul, South Korea); MET hydrochloride (Metfor) was obtained from Tabuk Pharmaceuticals (Tabuk, Saudi Arabia).

Animal Treatments

Male albino rats (n = 24; aged 10-12 weeks) were housed individually under a 12 h light/dark cycle (lights on 7:00 am) with free access to food and water. Animals were divided into four groups (control, CMF, MET, and CMF+ MET; n = 6 per group). After 2 weeks, rats were subjected to cognitive function evaluation. Rats were euthanized using carbon dioxide (CO_2), and brains were collected and stored at -80°C until analysis.

Drug Administration

Rats were injected intraperitoneally (i.p.). with CMF (50 mg/kg cyclophosphamide, 2 mg/kg methotrexate, 50 mg/kg fluorouracil, two doses over 2 weeks). However, metformin was dissolved in drinking water at 2.5 mg/mL and administered during the 2 weeks after the first CMF injection. Rats in the control group received two injections of saline.

Preparation of Brain Samples

Animals were euthanized with CO_2 and heads were decapitated prior to removing brains. Brains were washed with oxygenated phosphate-buffered saline (PBS) solution to remove blood. Neuronal lysis buffer (N-PER; Thermo Scientific, Madison, WI, USA) was used as a homogenizer. Samples

were centrifuged at 12,000 g for 10 min at 4°C, supernatants were transferred into new centrifuge tubes, and total protein was estimated by bicinchoninic acid (BCA) assay before running high-performance liquid chromatography (HPLC) experiments[2].

Fear Conditioning and Behavioral Assessment of Fear Memory

Twenty-four male albino rats (age 12-14 weeks, weight 250-300 g) were used in this study. Rats were placed in a standard rat operating chamber (housed in a sound-isolation cubicle), the grid floor of which could be electrified to deliver foot-shock (hereafter the context). Rats were trained with a conditioned freezing protocol. During fear conditioning, all rats were placed in context for 30 min on day 1 for habituation to the chamber without foot-shock. However, on day 2, rats were returned to the chamber for context for 180 s and animals received multiple electrical foot-shocks in different contexts. Twenty-four hours later (day 3), animals were returned to context for 180 s but without delivering electrical foot-shock. Freezing behavior (absence of all movements except for those related to respiration) was determined to evaluate fear memory function by analyzing differences in freezing time between treated groups[24].

HPLC Analysis of Glutamate

Quantitative HPLC was performed on a Waters-Alliance HPLC instrument (Waters) equipped with a photodiode-array detection (PDA) detector module, an automatic injector (injection volume 50 µL), a quadra-pump, and a Supelco C18 column (250 x 4.6 mm i.d, particle size 5 µm) as the stationary phase. The HPLC system was controlled by Empower-3 software. The column was maintained at 35°C and eluted under isocratic conditions over 10 min at a flow rate of 1 mL/min. Trifluoroacetic acid (TFA): tetrahydrofuran (THF) at a 90:10 v/v ratio was used as the mobile phase used to elute glutamate from brain samples after filtering through a 0.45 µm nylon membrane filter and degassing. UV detection was performed at 245 nm with a PDA detector, and 0.15% (v/v) TFA was used as diluent for the biological matrix to extract glutamate present in the sample. The retention time of glutamate was 2.9 min with a good symmetrical peak shape and a USP theoretical plate count >2000, hence peaks did not suffer from interference from endogenous compounds present in the rat brain sample matrix. The retention times, peak heights, and respective peak areas were repeatable, with RSD values <2.

HPLC Analysis of Dopamine

Quantitative HPLC was performed on a Waters-Alliance HPLC instrument (Waters) equipped with a PDA detector module, an automatic injector (injection volume 50 µL, a quadra-pump), and an Inertsil 3V ODS C18 column (250 x 4.6 mm i.d, particle size 5 µm) as the stationary phase. The HPLC system was controlled by Empower-3 software. The column was maintained at 35°C and eluted under isocratic conditions over 20 min at a flow rate of 1 mL/min. Potassium dihydrogen phosphate buffer (0.05 M) with the pH adjusted to 2.3 using ortho-phosphoric acid was used as the mobile phase to elute dopamine from brain samples after filtering through a 0.45 µm nylon membrane and degassing. UV detection was performed at 278 nm with a PDA detector. Deionized water served as diluent for the biological matrix dopamine present in the sample. The retention time of dopamine was 14.05 min with a good symmetrical peak shape and a USP theoretical plate count >2000, hence, peaks did not suffer from interference from endogenous compounds present in the rat brain sample matrix. The retention times, peak heights, and respective peak areas were repeatable, with RSD values <2.

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Results are presented as means ± standard error of the mean (SEM), and $p < 0.05$ was considered to indicate statistical significance (n = 6 experiments).

Results

Fear Conditioning and Behavioral Assessment of Fear Memory

To assess the effects of CMF and MET on fear conditioning, animals were treated with the CMF, MET, or CMF+MET, and subjected to fear conditioning memory analysis as described above. As shown in Figure 1, fear memory was observed in control and MET groups, as evidenced by a longer freezing behavior duration than CMF and CMF+MET groups. Thus, CMF and CMF+MET impaired fear memory compared with control and MET treatments.

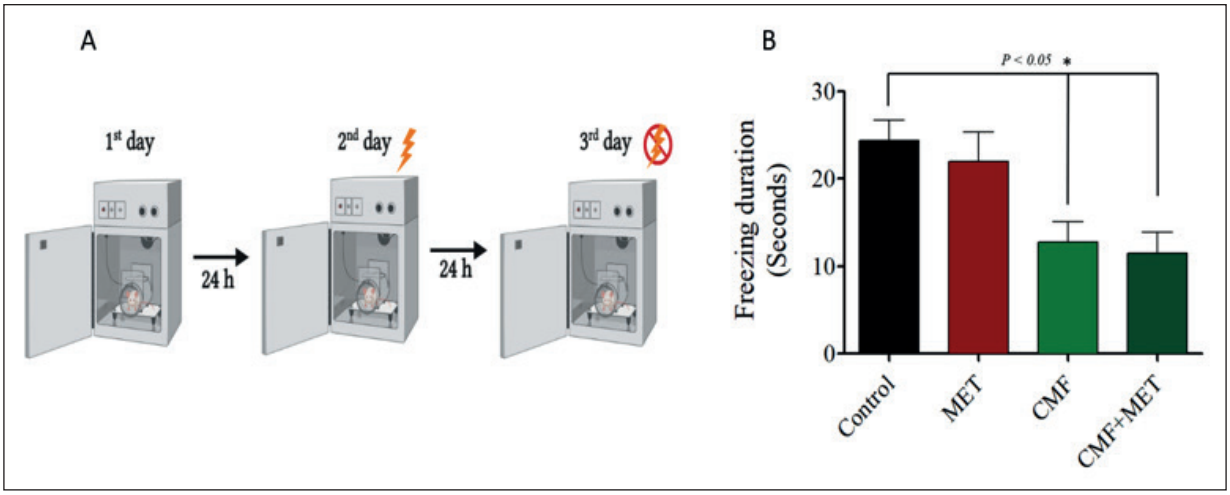

Figure 1. Effects of CMF and MET on fear context memory. **A**, Schematic diagram of the fear memory experimental setup. **B**, Effects of CMF and MET on freezing behavior duration following chemotherapy treatment. Data analysis was performed using Tukey's multiple comparison test (*p <0.05).

Evaluation of Glutamate Levels in the Brain Following CMF and MET Treatments

To evaluate the effects of CMF and MET on levels of glutamate, brain homogenates of CMF, MET, or CMF+MET were tested using HPLC and the results were compared with control non-treated animals. As shown in Figures 2 and 3, the levels of glutamate in CMF and CMF+MET were significantly increased compared with control group.

Evaluation of Dopamine Levels in the Brain Following CMF and MET Treatments

To evaluate the effects of CMF and MET on levels of dopamine, brain homogenates of CMF, MET, or CMF+MET were tested using HPLC and the results were compared with control non-treated animals. As shown in Figures 4 and 5, the levels of dopamine in MET, CMF, and CMF+MET were significantly increased compared with control group.

Discussion

In the present study, we investigated the effects of CMF and MET treatment on induced neuronal toxicity, such as memory impairment, and the effects of treatments on glutamate and dopamine levels in rat brains. The results showed that metformin failed to mitigate neurotoxicity and ameliorate memory impairment (Figure 2). However, we assessed fear conditioning memory, and levels of glutamate and dopamine were altered significantly (Figure 3 and 4), which could result in memory impairment. Our previous studies[25] using Y-maze, novel object recognition (NOR), and elevated plus maze (EPM) approaches revealed that CMF and MET can affect memory function. In the current study, we evaluated fear conditioning memory, which functions through a different pathway (the amygdala hippocampal pathway), as well as glutamate and dopamine in relation to memory function alteration.

The glutamatergic system is the major excitatory neurotransmitter system in the vertebrate brain[26]. Glutamate is a non-essential amino acid that is synthesized in the brain and binds to glutamate receptors, which plays a crucial role in regulating general brain functions, including memory formation and synaptic plasticity[13,27]. The two major ion channel glutamate receptors, N-methyl-D-aspartate receptors (NMDARs)[28] and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs)[29,30], are mainly involved in the induction of memory formation. When presynaptic neurons stimulate and release glutamate into the synaptic cleft, it binds to AMPARs and elicits an influx of Na^+, causing depolarization of postsynaptic neurons. At resting potential, NMDARs are blocked by Mg^{2+}, but depolarization of postsynaptic neurons caused by activation of AMPARs leads to removal of Mg^{2+} blocking NMDARs, allowing influx of Ca^{2+} and Na^+. Ca^{2+} can induce downstream signaling by activating calcium calmodulin-dependent kinase II/IV (CaMKII/CaM-

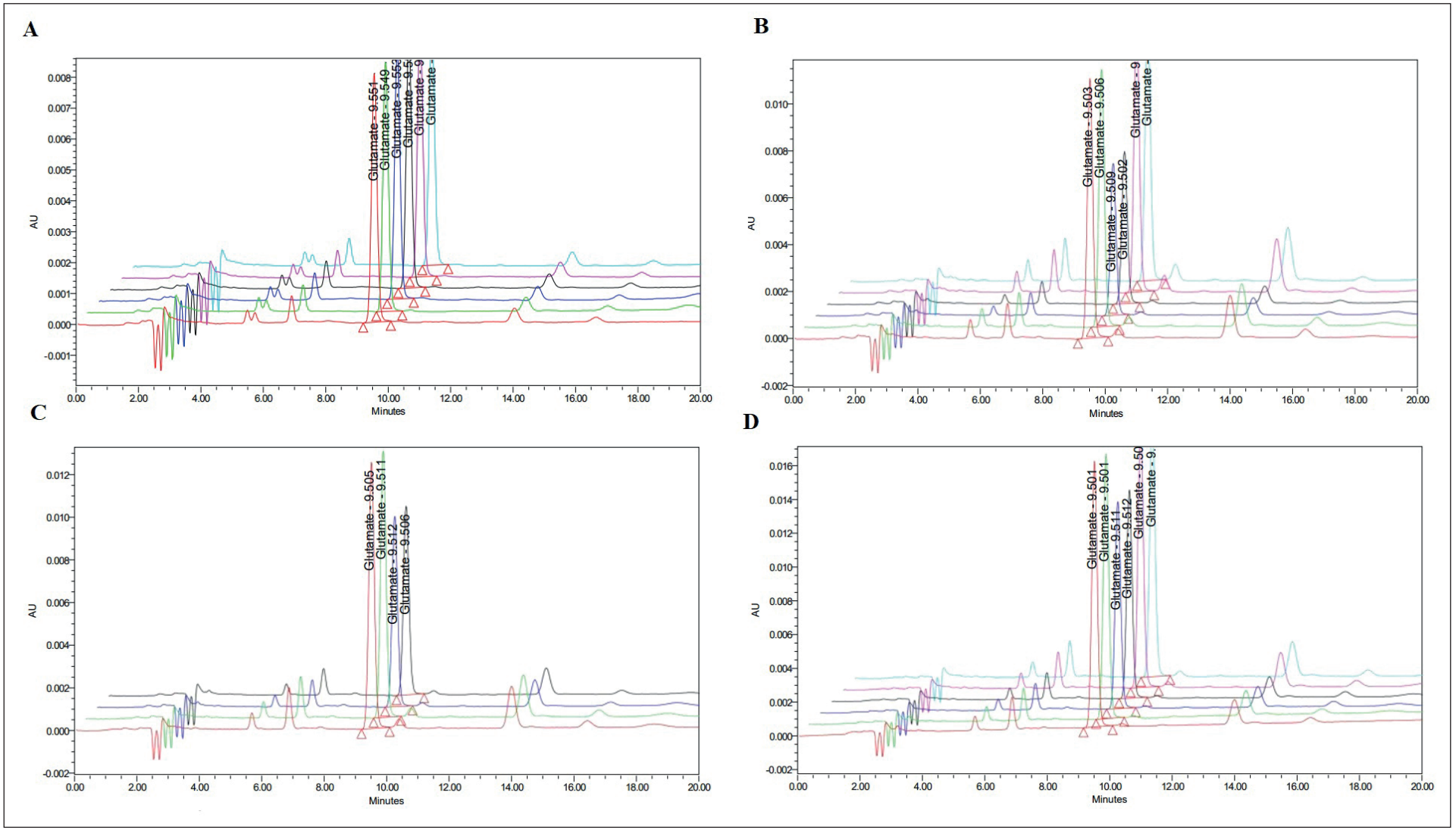

Figure 2. Chromatograms of glutamate levels in rat brain samples. **A**, Glutamate controls. **B,** Glutamate in MET-treated rat brain samples. **C**, Glutamate in CMF-treated rat brain samples. **D**, Glutamate in rat brain samples treated with MET + CMF.

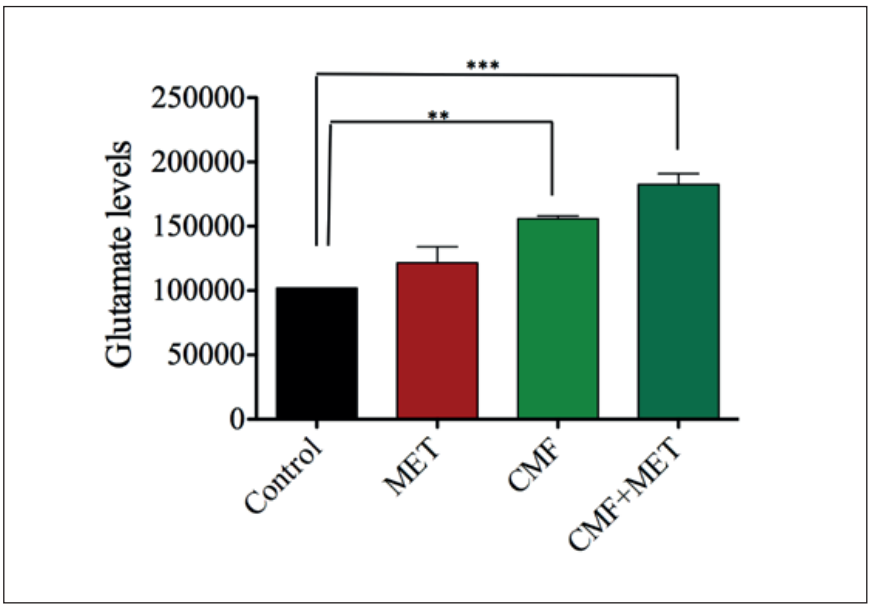

Figure 3. Glutamate levels in the brain are increased following treatment with CMF and CMF+MET. Data analysis was performed using Tukey's multiple comparison test (*p <0.05, **p <0.01, ***p <0.001).

KIV)[31]. This activation of CaMKII/CaMKIV leads to a complex downstream signaling cascade, leading to alteration of synaptic strength and memory processes[32]. By contrast, excessive activation of glutamate receptors can result in neuronal toxicities and cell death. Therefore, decreased or increased levels of Ca^{2+} can downregulate or upregulate AMPARs and NMDARs. Interestingly, our current study revealed that MET, CMF, and CMF+MET treatments markedly increased glutamate levels in the brain (Figures 2 and 3), which indicates a potential mechanism of memory impairment following MET and MET treatments.

Dopaminergic neurons mainly originate from the substantia nigra that innervate several regions of the brain[33]. Once dopamine is released, it binds to inhibitory or stimulatory dopaminergic receptors[33]. These receptors belong to the GPCR superfamily, and are distributed in many regions of the brain, including the hippocampus[34,35], which plays a major role in memory formation[30,36]. Dopamine receptors play essential roles in regulating learning and memory processes[37,38], and reducing dopamine levels in the brain can result in memory impairment[39,40]. In addition, excessive dopamine levels can cause overactivation of dopaminergic receptors, particularly type-1 receptors, ultimately leading to memory impairment[37,41]. The current study revealed that dopamine levels were markedly elevated in the brain following CMF, MET, and combined CMF+MET treatments (Figures 4 and 5), indicating a potential mechanism of memory impairment caused by chemotherapy.

Conclusions

In this study, we investigated the effects of CMF and MET treatments on inducing cognitive impairment, and we explored the mechanism underlying this cognitive impairment by measuring levels of glutamate and dopamine in the brain following CMF and MET treatment using chemobrain rat models. The results showed that CMF and combined CMF+MET treatments significantly reduced the freezing behavior duration in the context fear memory task, and this was associated with increased levels of glutamate and dopamine in the brain in MET, CMF, and CMF+MET groups. Therefore, we believe that these changes reflect the mechanism underpinning cognitive impairment following CMF and MET treatments. Further studies are necessary to elucidate the mechanisms of chemotherapy-induced cognitive impairment, which could shed light on the prevention of chemobrain, and assist the development of new strategies to treat this phenomenon.

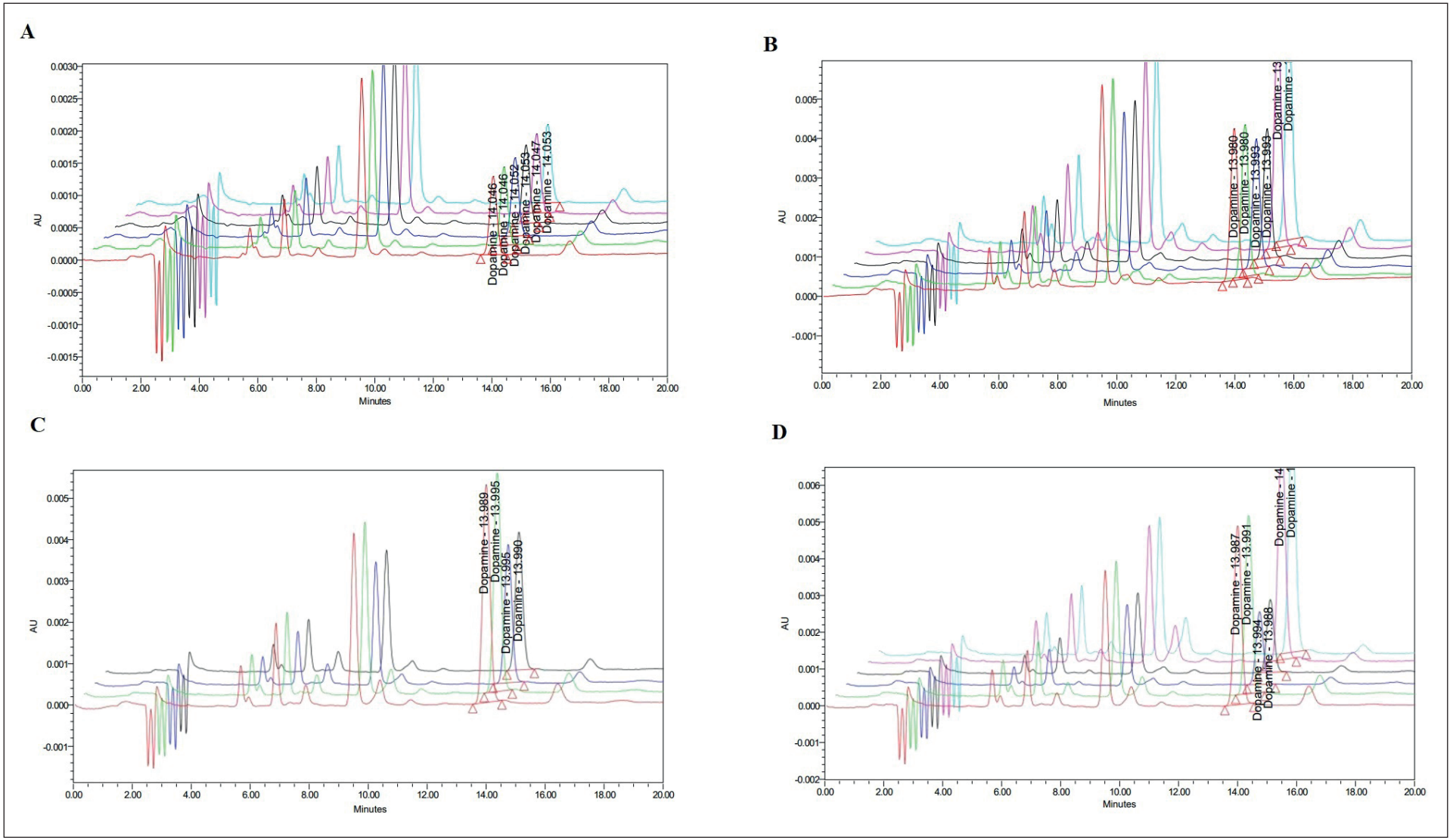

Figure 4. Chromatograms of dopamine levels in rat brain samples. **A**, Dopamine in controls. **B**, Dopamine in MET-treated rat brain samples. **C**, Dopamine in CMF-treated rat brain samples. **D**, Dopamine in rat brain samples treated with MET + CMF.

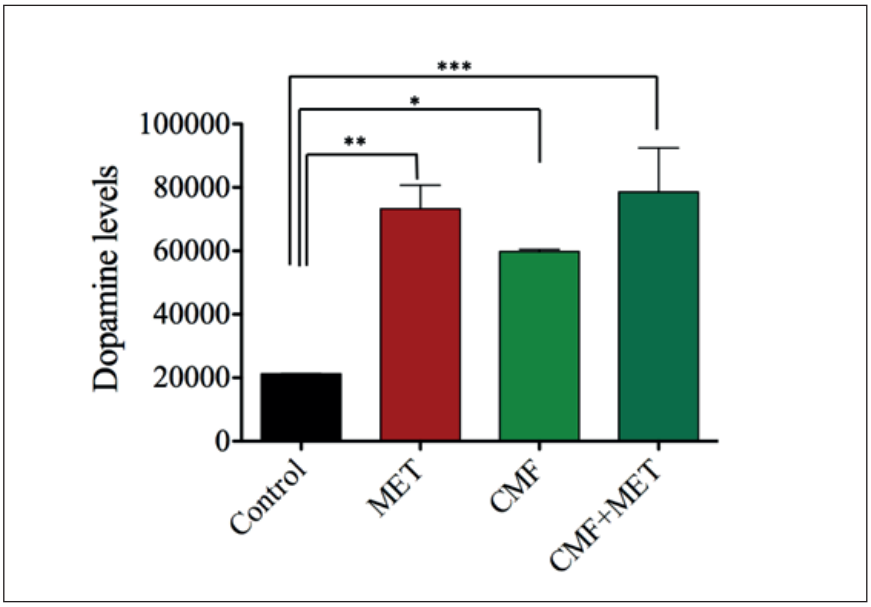

Figure 5. Dopamine levels in the brain are increased following treatment with MET, CMF, and CMF+MET. Data analysis was performed using Tukey's multiple comparison test (*p <0.05, **p <0.01, ***p <0.001).

Data Availability
All data are available upon request.

Conflicts of Interest
The authors declare no conflict of interest.

Ethical Approval
Animal studies were approved by the Deanship of Scientific Research, Qassim University (pharmacy-2019-2-2-I-5603).

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Authors' Contributions
A.A. and M.A. designed and performed experiments, analyzed, and interpreted the results, and prepared the manuscript. S.C. and R.N. contributed to HPLC experiments. Y.A. and A.A. (Ahmed Abdellatif) contributed to revising the manuscript. All authors read and agreed to the published version of the manuscript.

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