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Metformin's effect on male rats experiencing CMF-induced neurotoxicity

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Abstract. – OBJECTIVE: Numerous cancers are treated with the chemotherapy drugs cyclophosphamide (CP), methotrexate (MT), and fluorouracil (FU). However, it should be noted that neurotoxicity is a possible side effect of chemotherapy. The pharmaceutical agent metformin (MTF) is used to control type 2 diabetes. The administration of MTF has been documented to exhibit a reduction in specific toxic effects associated with chemotherapy. The primary purpose of this research was to examine whether MTF could mitigate the neurotoxicity brought on by cranial magnetic field (CMF).

MATERIALS AND METHODS: A cohort of forty male rats was divided into four distinct groups, with ten animals in each. We classified them as either saline, MTF, CMF, or CMF+MTF. The rats in the experiment group received two doses of CMF via intraperitoneal injection and were also given MTF in their drinking water at a concentration of 2.5 mg/mL on a daily basis. Brain tissue was obtained for ELISA of Bax, Bcl-2, and caspase-3 expression, as well as to determine NMDA and AMPA receptor mRNA expression by real-time polymerase chain reaction (RT-PCR) analysis.

RESULTS: Expression of AMPAR, NMDAR, Bax, Bcl-2, and caspase-3 was not notably different between the saline and MTF groups. In contrast, mRNA expression for AMPAR, NMDAR, Bax, and caspase-3 was notably upregulated in the CMF group, while Bcl-2 was downregulated. The co-administration of MTF and CMF did not mitigate these side effects.

CONCLUSIONS: neurotoxicity was induced in rats by CMF treatment, but the elevation of the glutamatergic system and the elevation of apoptotic proteins were not prevented by the MTF co-treatment.

Key Words:
Rats, Cyclophosphamide, Methotrexate, Fluorouracil, Metformin, Neurotoxicity, AMPA, NMDA, apoptosis.

Introduction

Chemotherapy exhibits notable efficacy against a diverse range of cancer types owing to its principal mode of action, which entails the induction of cytotoxicity[1]. The high efficacy exhibited by chemotherapy enables its application in the treatment of a diverse range of malignancies. It is plausible that the administration of chemotherapy may potentially induce adverse effects such as cardiotoxicity, nephrotoxicity, hepatotoxicity, hypothyroidism, and neurotoxicity in individuals undergoing treatment[2]. Unfortunately, current pharmacology lacks the ability to effectively treat the harmful effects associated with chemotherapy. Previous investigations have undeniably yielded fruitful outcomes in unveiling a noteworthy association between escalated concentrations of pro-inflammatory cytokines within the central nervous system, particularly interleukin-6 (IL-6) plus interleukin-1 beta (IL-1beta), thereby instigating neurotoxicity and cognitive deterioration[3]. Furthermore, it has been noted that exposure to CMF, a therapeutic protocol comprising cyclophosphamide (CP), methotrexate (MT), and 5-fluorouracil (FU), elicits an increase in cytokine concentrations, ultimately leading to the development of hypothyroidism and cardiotoxicity[4]. Accordingly, the primary aim of this investigation was to assess the inherent capabilities of pharmaceutical agent metformin (MTF) in mitigating the neurotoxic consequences elicited by CMF therapy. The determination was made by quantifying the mRNA expression levels of glutamate receptors, in conjunction with the quantification of pro-apoptotic and anti-apoptotic protein appearance levels.

Cyclophosphamide, Methotrexate, and Fluorouracil, known as CMF, represent a formidable amalgamation of chemotherapeutic agents that manifest a remarkable synergy in the therapeutic intervention of a diverse range of malignancies[5]. The mechanism of cyclophosphamide works by causing DNA alkylation, which ultimately leads to the inhibition of DNA transcription and RNA synthesis[6,7]. The abbreviation MT, denoting methotrexate, pertains to a pharmacotherapeutic agent

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that functions as a potent antagonist of folate[8,9]. The pharmacological mechanism of action involves the inhibition of multiple enzymes that are crucial for the biosynthesis of nucleotides[10]. The enzymatic conversion of dihydrofolate to tetrahydrofolate is a pivotal step in the intricate biochemical pathways that contribute in the synthesis of DNA and RNA nucleotides[11]. The dihydrofolate reductase (DHFR) enzyme assumes a critical role in this process, serving as a key facilitator. As an antimetabolite, fluorouracil (FU) exerts its inhibitory effects by effectively impeding the crucial process of thymidylate formation, which initiates with uracil[12,13]. As a result of this phenomenon, the intricate process of DNA and RNA synthesis is impeded, leading to a perturbation in the typical cellular activities of both molecules[14]. Furthermore, an abundance of literature has undeniably established that the application of CP, MT, and FU elicits a substantial reduction in neurogenesis within the cerebral region, concomitantly inducing hypothyroidism and manifesting cardiotoxic properties[4,15]. Recent research[4] findings have unveiled that the inadvertent consequences of these pharmacological agents possess the capacity to induce cognitive decline, alongside a decrement in cardiac performance. Hence, it is of utmost importance to undertake additional inquiries in order to elucidate the underlying mechanisms through which CMF elicits cognitive impairment.

Metformin, or MTF, is a pharmacotherapeutic agent categorized within the biguanide medication[16]. It is primarily specified for the management and treatment of diabetes mellitus[17]. The principal mechanisms through which glucose levels are reduced encompass the suppression of hepatic gluconeogenesis and the receptor augmentation of insulin sensitivity[18]. MTF has been widely employed for an extended period of time as an efficacious therapeutic intervention for the management of diabetes mellitus[19]. Metformin, being the foremost therapeutic option for diabetes mellitus, stands as the preeminent oral antihyperglycemic agent in extensive clinical practice[20]. Metformin has numerous additional health benefits beyond its well-known therapeutic uses in diabetes, polycystic ovary syndrome, cancer, and metabolic syndrome[21,22]. The efficacy of metformin in mitigating the adverse effects of chemotherapeutic agents and enhancing patient survival in rats was substantiated by various lines of evidence[23,24].

Recent investigations have shed light on the capacity of MTF to improve the adverse effects on neural tissue and reduce the harm inflicted on the brain by chemotherapeutic substances, namely cyclophosphamide, and cisplatin, in experimental models involving rodents[23,24]. Moreover, it was revealed that the rodents exposed to CMF intervention displayed signs of neurotoxicity, which can be identified by increased neuroinflammation. This is supported by the elevated levels of IL-1beta, tumor necrosis factor-alpha (TNF-α), and IL-6, besides the heightened concentrations of neurotransmitters dopamine and glutamate in the cerebral region[25,26]. Moreover, it has been noted that cranial magnetic field stimulation (CMF) exhibits the capacity to induce hypothyroidism and cardiotoxicity in experimental rodent models[4]. Regrettably, the simultaneous administration of MTF failed to demonstrate any therapeutic advantages on the aforementioned outcomes[4]. Furthermore, the neurons located in the hippocampus region were exposed to a condition known as CMF, which led to a distinguished decrease in the functioning of mitochondrial complex I[27]. The investigation was conducted to assess the potential therapeutic effectiveness of MTF as a possible intervention for the aforementioned mitochondrial dysfunctions[27]. The consequences obtained from this investigation provide compelling evidence that the administration of MTF does not demonstrate a statistically significant decrease in the neurotoxic effects elicited by CMF.

The central objective of this investigation is to elucidate the underlying mechanisms through which CMF treatment induces neurotoxicity, while also exploring the probable mitigating effects of MTF combination in ameliorating the harmful consequences associated with CMF.

Materials and Methods

Study Area

The inquiry was carried out at the renowned academic institution known as Qassim University, particularly the Pharmacy College, located in the KSA, during the period spanning from July 1st to July 30th, 2021.

Drugs

Cyclophosphamide (CP) was acquired from the esteemed pharmaceutical manufacturer Baxter (Mumbai, Maharashtra, India). MT, or methotrexate, was procured from Hospira UK Ltd., a renowned pharmaceutical establishment (Leeds, United Kingdom). Fluorouracil (FU) was procured from Korea Company (Seoul, South Ko-

rea). Metformin hydrochloride (MTF) was procured from Tabuk Pharma Company (Tabuk, Saudi Arabia).

Experimental Design

In order to conduct this investigation, forty albino male rats were acquired from the animal household situated within the Pharmacy College at Qassim University. The murine subjects were placed in enclosures that followed a predetermined light-dark cycle of 12 hours each, along with a carefully regulated ambient temperature ranging from 23 to 26 degrees Celsius. The perpetual absence of restrictions on the availability of sustenance and hydration was observed in their circumstances. The experimental subjects, in this case rodents, were partitioned into four distinct groups, referred to as cohorts, with each cohort comprising a total of ten individual rats. A control group was carefully chosen and subjected to the administration of a saline solution, while another group was administered MTF. The administration of the CMF regimen was assigned to the third cohort, whereas the fourth cohort received the CMF regimen in conjunction with MTF. MTF was intravenously administered as an aqueous solution, which was carefully prepared through the dissolution of the compound in potable water. The solution's concentration exhibited a consistent value of 2.5 milligrams per milliliter over the entirety of the administration period. The experimental subjects, in this case, were rats that underwent intraperitoneal administration (i.p.) of CMF, a combination of cyclophosphamide at a concentration of 50 mg/kg, methotrexate at a concentration of 2 mg/kg, and fluorouracil at a concentration of 50 mg/kg. Each administration of a dose was temporally spaced apart by a duration of two weeks. However, the administration of MTF in the CMF+MTF experimental group was carried out using an aqueous solution with a concentration of 2.5 milligrams per milliliter, following the preliminary injection of CMF (controlled microfluidics). In contrast, the control group of rodents received dual injections of a saline solution. After conducting daily observations on the rats' mortality rate and body weight in relation to the treatments they received, the subjects were then subjected to a biochemical evaluation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The hippocampal samples collected from both DOX-treated and control animals, and RNA extracted from them utilizing the TRIzol reagent, which was developed by Sigma-Aldrich (St. Louis, MI, USA). Following isolation of the total RNA, RNase-free DNase (Ambion, Carlsbad, CA, USA) was used to remove any remaining genomic DNA. Absorbance and concentration of RNA were determined with the help of a NanoDrop spectrophotometer from Thermo Fisher Scientific in Loughborough, UK. With a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA), we used total RNA (500 ng) to create a strand of complementary DNA (cDNA). Next, amplification of the cDNA samples was performed with Taq DNA polymerase (Qiagen, Shanghai, China). Specifically, the study made use of the fluorescent dye SYBR Green, which is widely used in the field of molecular biology, particularly in the field of RT-PCR. Bio-Rad, an established firm with headquarters in Hercules, California, USA, provided the iCycler iQ5 system used for the RT-PCR procedure. Primers were synthesized using an in-house program at Integrated DNA Technologies. Using the recommended settings for the RT-PCR experiment (30 seconds, an initial denaturation step at 95°C), the RT-PCR was carried out with Bio-Rad's Advanced SYBR Green Supermix. Forty amplification cycles then follow, with each cycle consisting of a 5-second denaturation at 95 degrees Celsius, followed by a 30-second annealing/extension at 57 degrees Celsius. Two identical samples were carefully made, and three tests were performed. The acquired data were put through automated processing with the AiraMx software for quantitative comparison once the plate-setting was complete. The expression of individual genes normalized with respect to *GAPDH*, a commonly used housekeeping gene. Transcript abundance per gene was estimated in comparison to *GAPDH* as a reference to quantify changes in mRNA expression (Table I).

ELISA

On the fourteenth day, after the ultimate administration of the pharmaceutical compound, the rodents were subjected to euthanasia *via* cervical decapitation subsequent to the initiation of anesthesia utilizing carbon dioxide (CO_2). The sample of blood was washed from the brains utilizing phosphate-buffered saline (PBS). The brain was homogenized using N-PER lysis buffer obtained from Thermo Scientific (Madison, WI, USA), followed by Qsonica homogenizer (operating at a frequency of 30 Hz) (Newtown, CT, USA). The samples were subjected to centrif-

Table I. Primers utilized in the investigation.

Gene	Sequence (5′–3′)	Length (bp)
GluA1	Forward: GCCAGATCGTGAAGCTAGAAA	80
GluA1	Reverse: CTCCGCTCTCCTTGAACTTATT	
NR2A	Forward: GGAGGAGGTTGGGTCATTTAT	86
NR2A	Reverse: AGTAGGCACTTGGGACTTTAC	
NR2B	Forward: GAGGAACCAGGCTACATCAAA	83
NR2B	Reverse: GGTCACCAGGTAAAGGTCATAG	
GAPDH	Forward: ACTCCCATTCTTCCACCTTTG	104
GAPDH	Reverse: CCCTGTTGCTGTAGCCATATT	

ugation at a temperature of 4.2°C for the temporal interval of 10 minutes, during which a force equivalent to 12,000 × g was applied. Following centrifugation, the resulting liquid portion above the sediment, known as the supernatant, was carefully transferred to newly prepared Eppendorf tubes. Before subjecting the samples to ELISA, the BCA assay was conducted in order to ascertain the overall protein content. The brain samples obtained from the rats belonging to both the CMF and control groups were subjected to ELISA analysis for the quantification of GluA1, Bax, Bcl-2, and caspase-3 levels. This analysis was performed using commercially available kits sourced from MyBioSource Company (San Diego, CA, USA). The experimental way followed the protocols provided by the manufacturers of the kits. The measurement of absorbance at a wavelength of 450 nm was performed utilizing a BIO-TEK Absorbance Microplate Reader, manufactured by BioTek (Winooski, VT, USA). The data were then subjected to statistical analysis.

Mitochondrial Complex I Activity

Brain tissues were subjected to homogenization using a mixture of phosphate-buffered saline (PBS) and lysis buffer (N-PERTM). The resulting homogenate was then subjected to centrifugation at a speed of 12,000 g and at 4°C for 10 minutes. The protein concentration in each sample was quantified through the utilization of the Bradford assay on the supernatant that was collected. The amount of complex I activity in mitochondria was determined by measuring the amount of NADH oxidized per milligram of protein using a spectrophotometric assay at 340 nm[27].

Lipid Peroxidation Assay

The collected rat brain tissues were homogenized in a lysis buffer known as N-PERTM. This homogenization process was then followed by centrifugation at a speed of 12,000 g for a duration of 10 minutes. The entire procedure was carried out at a temperature of 4°C. Upon the completion of the collection process, the supernatant was collected and carefully transferred into a sterile centrifuge tube. The bicinchoninic acid assay was employed to ascertain the approximate protein concentration of each sample prior to conducting the lipid peroxidation assay. Thiobarbituric acid, a spectrophotometric assay, was employed to quantify the extent of lipid peroxidation. The quantification of the lipid peroxidation index was conducted through the assessment of thiobarbituric acid-reactive substances (TBARS) generation at a wavelength of 532 nm. The generation of thiobarbituric acid reactive substances (TBARS) was observed to be dependent on the concentration of proteins, specifically measured as TBARS per milligram of protein[28].

Statistical Analysis

The data were implemented to a one-way ANOVA, and the results were summarized in terms of the mean standard deviation. These numbers were calculated using GraphPad (Boston, MA, USA). The Dunnett various comparison test was pragmatic to the data to assess the outcomes in relation to the control. A p-value of 0.05 was preferred as the cutoff point required to claim statistical significance.

Results

CMF Increases GluA-Containing AMPA Receptors, and MTF Did not Rescue

The ELISA findings demonstrated notable disparities in *GluA1* levels subunit mRNA and protein, exhibiting an elevation in the CMF experimental cohort. However, a rise in the GluA1 receptor subunit in CMF was observed when

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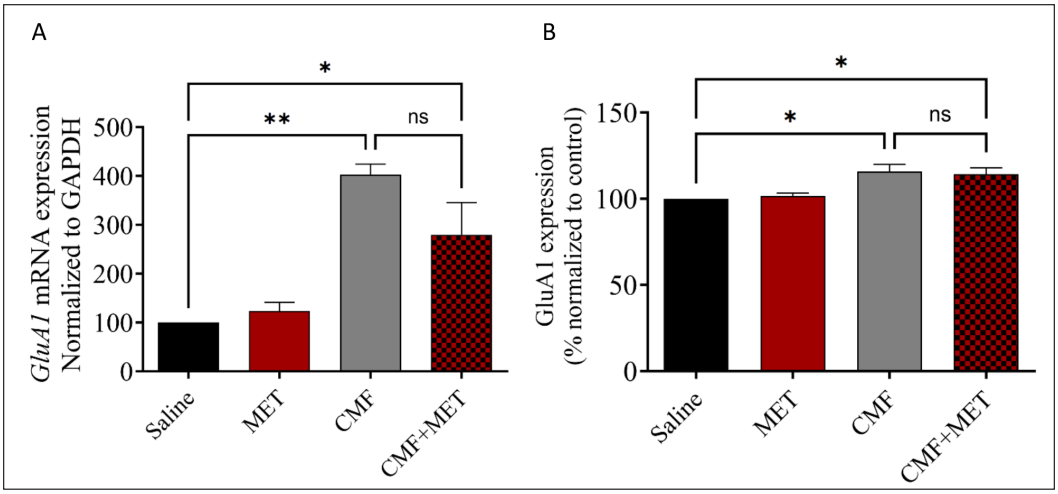

Figure 1. CMF and MTF co-administration effects on *GluA1*-containing AMPAR expression. **A**, Effects of CMF on mRNA expression of the *GluA1* subunit of AMPARs relative to that in control rats. **B**, Effects of CMF on hippocampal AMPAR GluA1 subunit protein expression relative to that in control rats. However, this increase in *GluA1* mRNA and GluA1 protein were changed by MTF co-administration. Bars indicate (*$p <$ 0.05, **$p < 0.01$) mean ± SEM.

combined with MTF in the CMF+ MTF experimental group, and this outcome remained unchanged (Figure 1).

CMF Increases NR2A and NR2B-Containing NMDA Receptors, and MTF did not Rescue

Based on the RT-PCR analysis results, a careful observation was made, indicating that the group exposed to CMF treatment exhibited a notable elevated concentration of *NR2A* and *NR2B* of NMDA receptors mRNA, in comparison to the rats administered with saline solution. The addition of the MTF combination to CMF did not alter the observed increase induced by CMF (Figure 2A-B).

MTF Did not Result in Any Significant Improvement in the Toxic Impacts of CMF on Bax, Bcl-2, and Caspase-3

The ELISA analysis demonstrated a significant upregulation in the concentrations of Bax and caspase-3, whereas Bcl-2 exhibited a prominent downregulation in the CMF group, comparable to the saline-administered rats. Nevertheless, it is imperative to display that the administration of MTF therapy in isolation did not exhibit a discernible impact on the expression of Bax, Bcl-2, and caspase-3 in comparison to the saline group treated with saline solution. The synchronized administration of MTF and CMF did not demonstrate any noticeable therapeutic augmentation, as indicated by the absence of observed amelioration in Figure 3A-C.

MTF Did not Result in an Amelioration of CMF's Deleterious Impact on Mitochondrial Functionality and Lipid Peroxidation

The biochemical analysis revealed a noticeable decrease in mitochondrial function concentration within the CMF group when compared to the saline rats. Conversely, it appears that the MTF therapy alone did not have a notable effect on the complex I of mitochondria or lipid peroxidation activity when compared to the saline group. The co-administration of MTF and CMF did not yield any discernible amelioration of the adverse effects induced by CMF (Figure 4A-B).

Discussion

This study sought to assess the effects of CMF intervention on neuronal function in rat models through the evaluation of mRNA expression of glutamate receptors, specifically AMPAR in the *GluA1* subunit, and NMDAR in the *NR2A* and *NR2B* subunits, in samples obtained from the brain. In addition, the hypothesis of this research was that the simultaneous administration of MTF and CMF could possibly have a protective effect

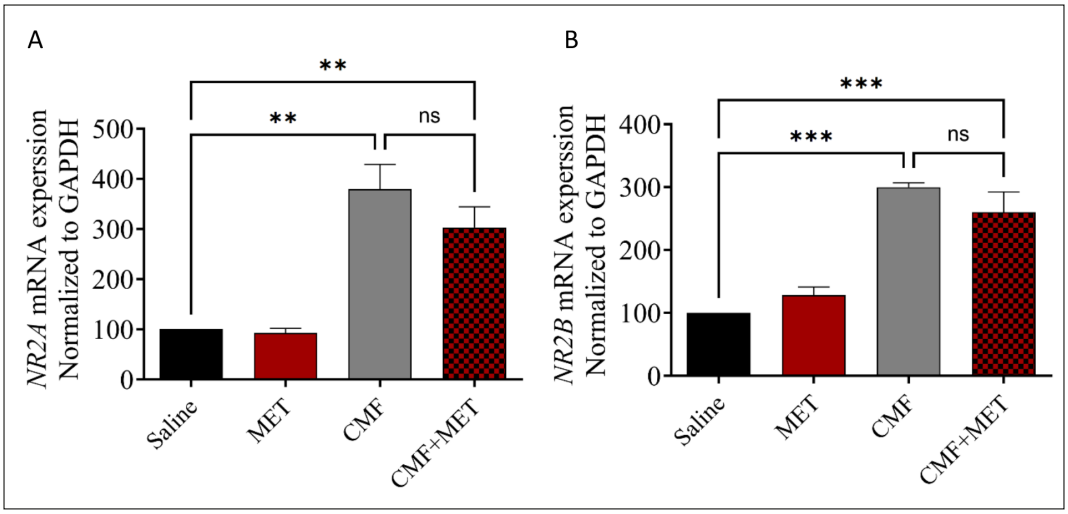

Figure 2. The impact of CMF and MTF co-administration on the mRNA expression of *NR2A* and *NR2B*, in comparison to the levels observed in control rats, is being evaluated. The expression of *NR2A* mRNA was observed to be significantly elevated in the CMF group in comparison to the control group. **A**, The expression of *NR2A* mRNA was observed to be significantly elevated in the CMF group in comparison to the control group. **B**, The rats that received CMF treatment displayed an increase in the expression of *NR2B* mRNA, analogous to the levels detected in the control rats. However, MET co-administration did not improve this increase in *NR2A* and *NR2B* mRNA. The utilization of bars in this context indicates the representation of the mean value along with SEM ($^{**}p < 0.01$, $^{***}p < 0.001$).

against the neurotoxicity caused by CMF. Nevertheless, it has been demonstrated that the administration of MTF in isolation does not induce any discernible modifications in neuronal function. Regrettably, the co-administration of MTF with CMF in rats did not manifest any protective effects against the neurotoxicity induced by CMF.

Glutamate is recognized as the primary excitatory neurotransmitter within the central nervous system (CNS) and assumes distinct roles in both physiological brain functioning and the development of diverse neurological disorders[29]. The pivotal role of glutamate receptors and transporters lies in their regulation of glutamate release and extracellular glutamate concentrations, thereby ensuring the maintenance of dynamic synaptic signaling processes and optimal memory function[30]. The prominent ion channel glutamate receptors encompass AMPA and NMDA receptors, both of which contribute to the intricate landscape of cognitive function[31]. In a concise manner, following the stimulation of presynaptic neurons, there is an augmentation in the release of glutamate[32]. This glutamate then proceeds to bind to both AMPA and NMDA receptors, thereby initiating the activation of various protein cascades[33]. Consequently, this process leads to the potentiation of memory formation[34]. Nevertheless, the excessive stimulation of AMPA and NMDA receptors by the neurotransmitter glutamate may result in an exaggerated influx of calcium ions, thereby inducing neuronal toxicity and degeneration[35].

Moreover, as demonstrated in our prior investigation, the administration of CMF therapy exhibited a notable impact on mortality rates and a reduction in body weight in rodent subjects[4]. In addition to the previously mentioned data, it has been elucidated that there is potential for CMF to induce neurotoxicity[27]. This sheds light on the toxicological effects of CMF and emphasizes the lack of effectiveness of MTF in reversing these alterations. The current study endeavors to evaluate the underlying mechanisms implicated in the neurotoxic effects prompted by CMF, along with the potential protective effects of MTF against these alterations. Consequently, the evaluation was performed on the functionality of the mitochondria and the proteins that initiate programmed cell death, specifically Bax, Bcl-2, and caspase-3, in addition to the assessment of lipid peroxidation.

The adequate expression and functionality of Bax and Bcl-2 are imperative for maintaining optimal cellular function and facilitating proper developmental processes[36]. Bax is a protein that exhibits pro-apoptotic characteristics, meaning it promotes programmed cell death. On the other hand, Bcl-2 is a protein that possesses anti-apoptotic properties, which means it inhibits or prevents programmed cell death[37]. Both Bax and Bcl-2 are integral components involved in the intricate

regulation of apoptotic pathways[38]. Bax protein plays a crucial role in promoting the process of pore formation in the mitochondrial membrane, which is a crucial event that facilitates the regulated release of cytochrome C[39]. Conversely, Bcl-2 exerts inhibitory effects on the formation of these pores[40]. Furthermore, it is noteworthy that the upregulation of Bax expression has the potential to induce the formation of pores in the mitochondrial membrane, leading to an excessive release of cytochrome C from the mitochondria[41]. This event subsequently triggers the beginning of apoptosis through the activation of caspase-3[42]. In the context of disease progression, it is noteworthy that the emergence of conditions such as Alzheimer's and Parkinson's has been observed to elicit an upregulation of Bax and a concomitant downregulation of Bcl-2[43,44]. This intricate molecular interplay ultimately culminates in the activation of caspase-3 expression, thereby instigating the process of neurodegeneration[45,46]. In a similar vein, it has been observed that certain chemotherapeutic agents, like cyclophosphamide, exhibit the ability to enhance Bax expression while concurrently reducing Bcl-2 expression, ultimately leading to the induction of apoptosis[47]. The findings of the present investigation unveiled notable changes in the expression levels of Bax and Bcl-2, as compared to the control group. Specifically, an upregulation of Bax and a downregulation of Bcl-2 were observed subsequent to CMF treatment. Hence, the administration of CMF therapy has the potential to augment the process of programmed cell death, known as apoptosis, through the upregulation of Bax expression, thereby triggering the activation of caspase-3 and subsequently initiating the apoptotic cascade. However, it has been hypothesized that the consumption of MTF could potentially result in the production of a protective effect on the productivity of CMF. The findings indicate that there was no alteration observed in the deregulation levels of CMF, suggesting that MTF did not exhibit protective effects against these effects.

Caspase-3 is a protease that belongs to the family of proteases that are specific for cysteinyl aspartate[48]. Caspase-3 is widely recognized for its

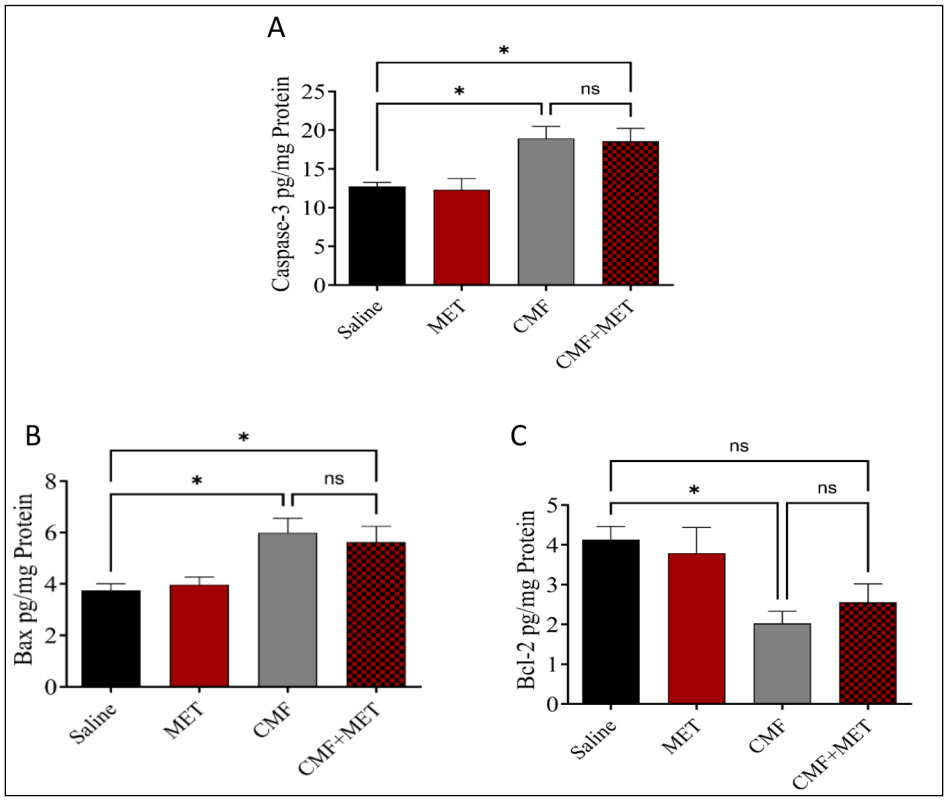

Figure 3. CMF and MTF affect Bax, Bcl-2, and caspase-3 levels. **A-C**, CMF treatment raised Bax and caspase-3 expression and diminished Bcl-2 expression relative to control rats, whereas MET treatment did not. MET co-administration did not inhibit the elevation in Bax and caspase-3 or elevate Bcl-2 levels induced by CMF treatment alone. *$p < 0.05$ related with the control using (a one-way ANOVA test).

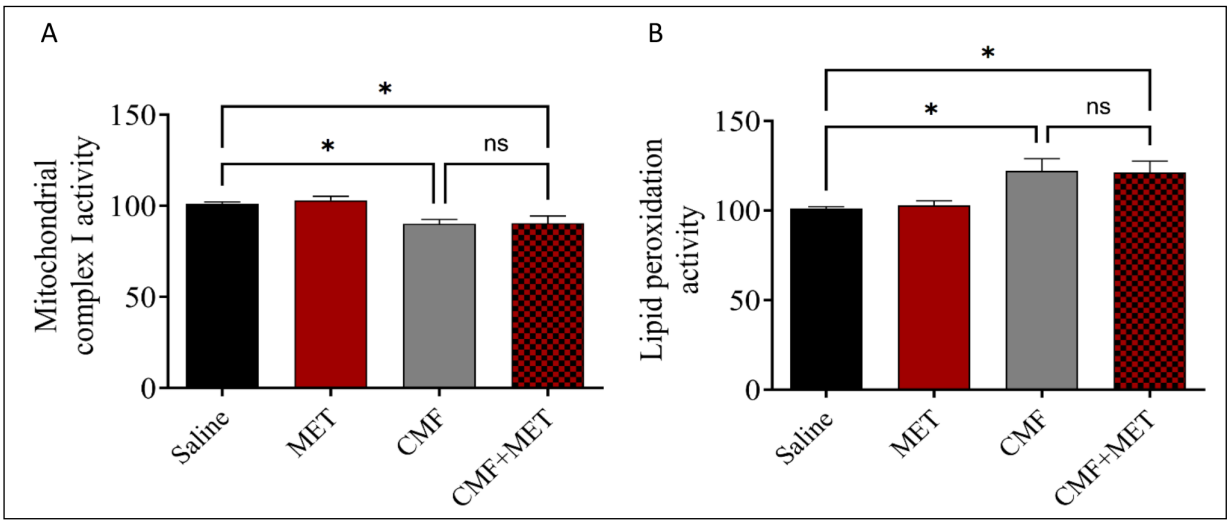

Figure 4. CMF, MTF, and combination impacts on mitochondrial complex I activity and lipid peroxidation activity in hippocampal tissues. The CMF therapy alone significantly declined the complex I activity (*$p < 0.05$). It elevated the lipid peroxidation activity, and CMF+MET did not improve the mitochondrial complex I activity (**A**) or lipid peroxidation activity (**B**) related to the rats in control (*$p < 0.05$) CMF related to the rats in control.

pivotal involvement in the intricate process of cellular apoptosis regulation[49]. It is also acknowledged to possess non-apoptotic functionalities[49]. One of the non-apoptotic functions of caspase-3 involves its participation in synaptic plasticity mechanisms that are essential for the process of memory formation[50]. In this study, it is analyzed the expression of caspase-3 in hippocampal cells of rat brains following treatment with CMF and MTF. The findings indicate that hippocampal neurons, when exposed to CMF, exhibited an elevated level of caspase-3 expression in comparison to the saline and MTF groups. In addition to its function in the regulation of the processes that underlie cellular apoptosis, caspase-3 exhibits the capacity to modulate synaptic plasticity and long-term depression through its involvement in the internalization of AMPA receptors. Based on the current findings, it can be observed that the administration of CMF treatment resulted in an upregulation of caspase-3 expression in the hippocampal tissues. This increase in caspase-3 levels may potentially contribute to the impairments in cognitive function by inducing neuronal apoptosis. However, it is noteworthy that the administration of MTF was unable to reverse this elevation in caspase-3 expression.

The present investigation exhibits certain notable strengths and limitations. The dosage employed in this investigation was of clinical relevance, mirroring the dosage administered to human cancer patients as well as the dosages utilized in other experimental literature. Henceforth, the outcomes may hold significance for individuals afflicted with cancer. The animal subjects utilized in this investigation were of identical strain and age, and all experimental procedures were executed concurrently across the study cohorts in order to mitigate the influence of potential confounding factors. Additionally, the direct effects of the CMF and MTF treatments were assessed in cancer-free rats, thereby eliminating any potential interference from cancer-related effects.

Conclusions

In summary, the outcomes of this investigation offer compelling evidence signifying that CMF therapy may potentially contribute to the emergence of neurotoxicity by promoting neuronal apoptosis *via* the upregulation of AMPA receptor protein and mRNA expression, along with increased expression of NMDA receptor mRNA. The observed enhancements in CMF-treated rodents were concomitant with a notable upregulation of Bax, Bcl-2, and caspase-3 within neuronal cells. Notably, the co-administration of metformin did not ameliorate this upregulation.

Authors' Contributions

A.H.A. and M.A.A made equal contributions in all aspects of the project, including conceptualization, study design, experiment conduct, data analysis, and manuscript writing.

Funding
None.

Ethics Approval
This study has been approved by the Qassim University Deanship of Scientific Research's Institutional Care of Animals Committee (approved number: pharmacy-2019-2-2-I-5603).

Informed Consent
Not applicable.

Data Availability
The relevant information supporting the findings of this study can be obtained from the corresponding author upon a reasonable request.

Conflict of Interest
The authors declare that they have no conflict of interests.

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References

1) G MS, Swetha M, Keerthana CK, Rayginia TP, Anto RJ. Cancer Chemoprevention: A Strategic Approach Using Phytochemicals. Front Pharmacol 2022; 12: 809308.
2) Trapani D, Zagami P, Nicolò E, Pravettoni G, Curigliano G. Management of Cardiac Toxicity Induced by Chemotherapy. J Clin Med 2020; 9: 2885.
3) Elgharabawy RM, Alhowail AH, Emara AM, Aldubayan MA, Ahmed AS. The impact of chicory (Cichoriumintybus L.) on hemodynamic functions and oxidative stress in cardiac toxicity induced by lead oxide nanoparticles in male rats. Biomed Pharmacother 2021; 137: 111324.
4) Alhowail AH, Aldubayan MA. The Impact of Metformin on the Development of Hypothyroidism and Cardiotoxicity Induced by Cyclophosphamide, Methotrexate, and Fluorouracil in Rats. Pharmaceuticals 2023; 16: 1312.
5) Park JH, Im SA, Byun JM, Kim KH, Kim JS, Choi IS, Kim HJ, Lee KH, Kim TY, Han SW, Oh DY, Kim TY. Cyclophosphamide, Methotrexate, and 5-Fluorouracil as Palliative Treatment for Heavily Pretreated Patients with Metastatic Breast Cancer: A Multicenter Retrospective Analysis. J Breast Cancer 2017; 20: 347-355.
6) Voelcker G. The Mechanism of Action of Cyclophosphamide and Its Consequences for the Development of a New Generation of Oxazaphosphorine Cytostatics. Sci Pharm 2020; 88: 42.
7) Gourzones C, Bret C, Moreaux J. Treatment May Be Harmful: Mechanisms/Prediction/Prevention of Drug-Induced DNA Damage and Repair in Multiple Myeloma. Front Genet 2019; 10: 861.
8) Koźmiński P, Halik PK, Chesori R, Gniazdowska E. Overview of Dual-Acting Drug Methotrexate in Different Neurological Diseases, Autoimmune Pathologies and Cancers. Int J Mol Sci 2020; 2: 3483.
9) Strober BE, Menon K. Folate supplementation during methotrexate therapy for patients with psoriasis. J Am Acad Dermatol 2005; 53: 652-659.
10) Friedman B, Cronstein B. Methotrexate mechanism in treatment of rheumatoid arthritis. Joint Bone Spine 2019; 86: 301-307.
11) S. Askari B, Krajinovic M. Dihydrofolate Reductase Gene Variations in Susceptibility to Disease and Treatment Outcomes. Current Genomics 2010; 11: 578-583.
12) Azwar S, Seow HF, Abdullah M, Faisal Jabar M, Mohtarrudin N. Recent Updates on Mechanisms of Resistance to 5-Fluorouracil and Reversal Strategies in Colon Cancer Treatment. Biology (Basel) 2021; 10: 854.
13) Sethy C, Kundu CN. 5-Fluorouracil (5-FU) resistance and the new strategy to enhance the sensitivity against cancer: Implication of DNA repair inhibition. Biomed Pharmacother 2021; 137: 111285.
14) Krump NA, You J. Molecular mechanisms of viral oncogenesis in humans. Nat Rev Microbiol 2018; 16: 684-698.
15) John J, Kinra M, Mudgal J, Viswanatha GL, Nandakumar K. Animal models of chemotherapy-induced cognitive decline in preclinical drug development. Psychopharmacology 2021; 238: 3025-3053.
16) Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia 2017; 60: 1577-1585.
17) Rojas LBA, Gomes MB. Metformin: an old but still the best treatment for type 2 diabetes. Diabetol Metab Syndr 2013; 5: 6.
18) Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiological Reviews 2018; 98: 2133-2223.
19) Drzewoski J, Hanefeld M. The Current and Potential Therapeutic Use of Metformin—The Good Old Drug. Pharmaceuticals (Basel) 2021; 14: 122.
20) Ahmad E, Sargeant J, Zaccardi F, Khunti K, Webb D, Davies M. Where Does Metformin Stand in Modern Day Management of Type 2 Diabetes? Pharmaceuticals 2020; 13: 427.
21) Lv Z, Guo Y. Metformin and Its Benefits for Various Diseases. Front Endocrinol 2020; 11: 191.
22) Lashen H. Review: Role of metformin in the management of polycystic ovary syndrome. Ther Adv Endocrinol Metab 2010; 1: 117-128.

23) Alhowail AH, Chigurupati S, Sajid S, Mani V. Ameliorative effect of metformin on cyclophosphamide-induced memory impairment in mice. Eur Rev Med Pharmacol Sci 2019; 23: 9660-9666.

24) Borchelt DR, Zhou W, Kavelaars A, Heijnen CJ. Metformin Prevents Cisplatin-Induced Cognitive Impairment and Brain Damage in Mice. Plos One 2016; 11: e0151890.

25) Alhowail AH, Aldubayan M. Recent progress in the elucidation of the mechanisms of chemotherapy-induced cognitive impairment. Eur Rev Med Pharmacol Sci 2021; 25: 5807-5817.

26) Alhowail AH, Almogbel YS, Abdellatif AAH, Aldubayan MA, Chigurupati S, Nemala RA. Effects of CMF and MET on glutamate and dopamine levels in the brain, and their impact on cognitive function. Eur Rev Med Pharmacol Sci 2022; 26: 2353-2362.

27) Alhowail AH, Almogbel YS, Abdellatif AAH, Aldubayan MA, Almami IS. Elucidating the mechanism underlying cognitive dysfunction by investigating the effects of CMF and MET treatment on hippocampal neurons. Eur Rev Med Pharmacol Sci 2022; 26: 743-749.

28) Bhattacharya D, Fujihashi A, Majrashi M, Bloemer J, Bhattacharya S, Buabeid M, Escobar M, Moore T, Suppiramaniam V, Dhanasekaran M. Concurrent nicotine exposure to prenatal alcohol consumption alters the hippocampal and cortical neurotoxicity. Heliyon 2020; 6: e03045.

29) Köles L, Kató E, Hanuska A, Zádori ZS, Al-Khrasani M, Zelles T, Rubini P, Illes P. Modulation of excitatory neurotransmission by neuronal/glial signalling molecules: interplay between purinergic and glutamatergic systems. Purinergic Signal 2015; 12: 1-24.

30) Gonçalves-Ribeiro J, Pina CC, Sebastião AM, Vaz SH. Glutamate Transporters in Hippocampal LTD/LTP: Not Just Prevention of Excitotoxicity. Front Cell Neurosci 2019; 13: 357.

31) Hansen KB, Wollmuth LP, Bowie D, Furukawa H, Menniti FS, Sobolevsky AI, Swanson GT, Swanger SA, Greger IH, Nakagawa T, McBain CJ, Jayaraman V, Low CM, Dell'Acqua ML, Diamond JS, Camp CR, Perszyk RE, Yuan H, Traynelis SF, Barker E. Structure, Function, and Pharmacology of Glutamate Receptor Ion Channels. Pharmacol Rev 2022; 73: 1469-1658.

32) Bialecki J, Werner A, Weilinger NL, Tucker CM, Vecchiarelli HA, Egaña J, Mendizabal-Zubiaga J, Grandes P, Hill MN, Thompson RJ. Suppression of Presynaptic Glutamate Release by Postsynaptic Metabotropic NMDA Receptor Signalling to Pannexin-1. J Neurosci 2020; 40: 729-742.

33) Vieira M, Yong XLH, Roche KW, Anggono V. Regulation of NMDA glutamate receptor functions by the GluN2 subunits. J Neurochem 2020; 154: 121-143.

34) Dong Z, Han H, Li H, Bai Y, Wang W, Tu M, Peng Y, Zhou L, He W, Wu X, Tan T, Liu M, Wu X, Zhou W, Jin W, Zhang S, Sacktor TC, Li T, Song W, Wang YT. Long-term potentiation decay and memory loss are mediated by AMPAR endocytosis. J Clin Invest 2014; 125: 234-247.

35) Verma M, Lizama BN, Chu CT. Excitotoxicity, calcium and mitochondria: a triad in synaptic neurodegeneration. Transl Neurodegener 2022; 11: 3.

36) Maes ME, Schlamp CL, Nickells RW. BAX to basics: How the BCL2 gene family controls the death of retinal ganglion cells. Prog Retin Eye Res 2017; 57: 1-25.

37) Hatok J, Racay P. Bcl-2 family proteins: master regulators of cell survival. Biomol Concepts 2016; 7: 259-270.

38) Qian S, Wei Z, Yang W, Huang J, Yang Y, Wang J. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. Front Oncol 2022; 12: 985363.

39) Zhang M, Zheng J, Nussinov R, Ma B. Release of Cytochrome C from Bax Pores at the Mitochondrial Membrane. Sci Rep 2017; 7: 2635.

40) Hardwick JM, Soane L. Multiple Functions of BCL-2 Family Proteins. Cold Spring Harbor Perspectives in Biology 2013; 5: a008722-a008722.

41) Liu Z, Ding Y, Ye N, Wild C, Chen H, Zhou J. Direct Activation of Bax Protein for Cancer Therapy. Med Res Rev 2016; 36: 313-341.

42) Parrish AB, Freel CD, Kornbluth S. Cellular Mechanisms Controlling Caspase Activation and Function. Cold Spring Harb Perspect Biol 2013; 5: a008672-a008672.

43) Abu-Elfotuh K, Tolba AMA, Hussein FH, Hamdan AME, Rabeh MA, Alshahri SA, Ali AA, Mosaad SM, Mahmoud NA, Elsaeed MY, Abdelglil RM, El-Awady RR, Galal ERM, Kamal MM, Elsisi AMM, Darwish A, Gowifel AMH, Mahran YF. Anti-Alzheimer Activity of Combinations of Cocoa with Vinpocetine or Other Nutraceuticals in Rat Model: Modulation of Wnt3/β-Catenin/GSK-3β/Nrf2/HO-1 and PERK/CHOP/Bcl-2 Pathways. Pharmaceutics 2023; 15: 2063.

44) Guo YL, Wei XJ, Zhang T, Sun T. Molecular mechanisms of melatonin-induced alleviation of synaptic dysfunction and neuroinflammation in Parkinson's disease: a review. Eur Rev Med Pharmacol Sci 2023; 27: 5070-5082.

45) Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. Nat Rev Mol Cell Biol 2019; 20: 175-193.

46) Mani V, Arfeen M, Ali HM, Hafez Abdel-Moneim AM, Aldubayan M, Dhanasekaran M, Alhowail A. Ciproxifan attenuates the memory impairment induced by lipopolysaccharide through modulation of cholinergic transmission in the mouse brain. Eur Rev Med Pharmacol Sci 2022; 26: 1897-1905.

47) Asiri YA. Probucol Attenuates Cyclophosphamide-induced Oxidative Apoptosis, p53 and Bax Signal Expression in Rat Cardiac Tissues. Oxid Med Cell Longev 2010; 3: 308-316.

48) Asadi M, Taghizadeh S, Kaviani E, Vakili O, Taheri-Anganeh M, Tahamtan M, Savardashtaki A. Caspase-3: Structure, function, and biotechnological aspects. Biotechnol Appl Biochem 2021; 69: 1633-1645.

49) Ponder KG, Boise LH. The prodomain of caspase-3 regulates its own removal and caspase activation. Cell Death Discov 2019; 5: 56.

50) D'Amelio M, Sheng M, Cecconi F. Caspase-3 in the central nervous system: beyond apoptosis. Trends Neurosci 2012; 35: 700-709.