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CMF and MET treatment induce cognitive impairment through upregulation of IL-1α in rat brain

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Abstract. – OBJECTIVE: Cyclophosphamide (CYP), methotrexate (MTX) and 5-fluorouracil (5-FU) (CMF) are chemotherapeutic agents known to cause acute and long-term cognitive impairment in cancer patients. Cognitive function is regulated mainly by neuronal circuitry in the brain, especially the cortex and hippocampus as well as other components of the limbic area. Neuroinflammation mediated by proinflammatory cytokines is a well - known cause of cognitive impairment. Our previous study showed that metformin induced cognitive impairment and neuroinflammation in CMF-treated rats. Understanding the effects and mechanisms of CMF and MET treatment on chemotherapy-related cognitive impairment and the relationship with neuroinflammation may help prevent some of the adverse effects of this type of chemotherapy in cancer patients.

MATERIALS AND METHODS: Rats were divided into four groups: control (normal saline), CMF (50 mg/kg CYP, 2 mg/kg MTX, 50 mg/kg 5-FU; two doses administered by intraperitoneal injection over two weeks), MET (2.5 mg/ml – oral administration daily), and CMF+MET group. IL-1α, IRS-1, Akt-a and TNF-α levels in brain tissues were measured by ELISA and data were analyzed by one-way ANOVA test followed by Tukey's test.

RESULTS: Compared with the control group, IL-1α levels were significantly increased in the CMF+MET group, whereas there were no significant differences in the MET and CMF groups. On the other hand, IRS-1, TNF-α and Akt-a expression and mitochondrial complex 1 activity indicated that systemic CMF and MET treatment did not change the expression of these proteins in the brain compared to the control group.

CONCLUSIONS: Our results indicate that cognitive function is impaired by the administration of two doses of CMF and MET over a period of two weeks as a result of IL-1α overexpression in the brain.

Key Words:
Chemotherapy, CMF, Cognitive impairment, Metformin, Mitochondrial function.

Introduction

Chemotherapy is one of the main strategies implemented to treat cancer. Despite its effectiveness against several types of malignancies, it can also affect normal tissues by inducing oxidative stress and inflammatory responses, leading to many side-effects and toxicities[1,2]. These side-effects can limit the clinical endpoints and reduce patients' quality of life[3,4]. Chemo-brain (also known as chemotherapy-related cognitive impairment) refers to a set of changes in cognitive functions that occur as a result of chemotherapy treatment[5]. It is characterized by deficits in patients' concentration, memory, decision-making, learning, and language during and after cessation of chemotherapy[6]. The signs of chemo-brain are

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usually temporary, although about 35% of clinically treated patients claim that the signs persist for months, or even years after discontinuation of the therapy[7]. Cognitive function is regulated mainly by neuronal circuitry in the brain, especially the cortex and hippocampus as well as other components of the limbic area. However, the underlying mechanism of cognitive dysfunction remains uncertain, but several pathways have been suggested, including both direct neurotoxicity of chemotherapeutic agents, and indirect oxidative stress, immune dysregulation, proinflammatory cytokine production, and blood-brain barrier (BBB) damage[8].

Cyclophosphamide (CYP), methotrexate (MTX), and 5-fluorouracil (5-FU) (CMF) are chemotherapeutic agents commonly used in combination to treat breast cancer[9,10]. CYP exerts its effects through the alkylation of DNA, thereby blocking DNA synthesis and RNA transcription[11]. MTX is a folate antagonist agent that inhibits several enzymes responsible for nucleotide synthesis, including dihydrofolate reductase, which catalyzes the conversion of dihydrofolate into tetrahydrofolate[1]. Tetrahydrofolate is essential for the synthesis of the DNA and RNA nucleotides[12]. As an antimetabolite, 5-FU inhibits the formation of thymidylate from uracil, thereby preventing DNA and RNA synthesis[13]. The BBB provides protection against cytotoxic agents, including chemotherapeutic medication[14]. Although many chemotherapeutic agents are unable to cross the BBB[15], some studies have demonstrated the ability of 5-FU and MTX to cross this barrier[16,17]. Furthermore, several studies have shown that the administration of MTX and CYP decreases neurogenesis in the brain, leading to impairment of learning and memory[17]. A previous clinical study of breast cancer patients treated with CMF about 21 years earlier showed that these individuals performed worse in several cognitive tests compared to those in the non-cancer control group[18]. Furthermore, one study showed that CMF-induced learning and memory impairment in rats was associated with a decrease in cellular proliferation in the hippocampus three weeks after chemotherapy[19].

Metformin (MET) is an oral agent that is commonly used as a first-line therapy for type 2 diabetes mellitus (T2DM)[20]. Besides treatment of T2DM, it is also used to treat other disorders such as obesity, metabolic syndrome, and polycystic ovarian syndrome[21]. MET stimulates the 5'-adenosine monophosphate-activated protein kinase (AMPK) pathway, which functions as a master regulator of catabolism and cell signaling pathways[22]. By inducing changes in the AMP:ATP ratio, AMPK inhibits gluconeogenesis in the liver[23]. AMPK activation is thought to be responsible for the anti-inflammatory and anticancer properties of MET[20,21]. The mammalian target of rapamycin (mTOR), a kinase that plays a critical in cellular growth, is inhibited by AMPK activation[24], resulting in blockade of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway[25]. As a pro-survival pathway, inhibition of the PI3K/Akt pathway promotes apoptosis[26]. It has been reported that diabetic patients treated with MET have a lower risk of cancer development than other diabetic patients treated using different medications[27]. Several studies demonstrated that MET acts synergistically with other chemotherapeutic agents in the treatment of cancer[28-30]. MET has also been shown to prevent memory impairment induced by MTX in the rat[31]. In addition, several studies indicated that MET treatment is associated with a lower risk of cognitive impairment in diabetic patients via AMPK-dependent and -independent mechanisms[32-34]. However, it is well known that long-term MET treatment is associated with vitamin B_{12} deficiency[35], which may exacerbate central nervous system disorders. Furthermore, the adjustment of vitamin B_{12} levels by supplementation is associated with better cognitive outcomes in diabetic patients experiencing vitamin B_{12} deficiency due to metformin treatment[36]. Our previous study showed that CMF and metformin treatment induced cognitive impairment by using hippocampal-dependent tasks such as Y-maze, novel object recognition and elevated plus maze through modulation of IL-6 in rats models of chemobrain [Alhowail AH, Almogbel Y, Abdellatif AAH, Aldubayan MA, Alfheeaid HA, Felemban SG, Chigurupati S, Alharbi IF, Alharbi HS. Metformin Induced Cognitive Impairment and Neuroinflammation in CMF-Treated Rats. Int J Pharmacol 2021 (in press)]. Based on these findings, we investigated the further effects of MET and CMF on cognitive function by evaluating the expression of the neuroinflammatory cytokines interleukin-1 alpha (IL-1α) and tumor necrosis factor-alpha (TNF-α) in the brain as well as the levels of insulin receptor substrate-1 (IRS-1) and protein kinase B (Akt-a). We also evaluated mitochondrial function by measuring complex 1 activity.

Materials and Methods

Drugs

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); and MET hydrochloride (Metfor®) was obtained from Tabuk Pharmaceuticals (Tabuk, Saudi Arabia).

Animals

Male rats (n = 24; aged 10–12 weeks) were housed individually under a 12-h light/dark cycle (lights on 6:00 am) with free access to food and water. The animals were observed daily to check the mortality rate, and their body weights were measured every two days. The animals were divided into the following four groups (n = 6 per group): control group, CMF group, MET group, and CMF+ MET group. At the end of the study period and after evaluation of cognitive function, the rats were euthanized using carbon dioxide (CO_2), and their brains were collected and stored in the -80°C until analysis. The Ethics Committee Approval was approved by the Deanship of Scientific Research, Qassim University under grant number (pharmacy-2019-2-2-I-5603), in accordance with the National Research Council (US) Guide for the Care and Use of Laboratory Animals.

Drug Administration

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

Enzyme-Linked Immunosorbent Assay (ELISA)

Rat brains were lysed with Neuronal Protein Extraction Reagent (N-PER™ obtained from Thermo Scientific™, Paisley, UK) and sonicated Qsonica homogenizer, 30 Hz pulses for 20 seconds (Qsonica, Newtown, CT, USA). After centrifugation at 12,000 ´*g* for 10 min, the supernatant was collected and aliquots of 200 µL were stored at -80°C. The protein content of each sample was quantified by BCA assay (Pierce, Waltham, MA, USA) and the concentrations of interleukin-1a, Akt-a, IRS-1, and TNF-a were analyzed using ELISA kits (Mybiosource Inc., San Diego, CA, USA) according to the manufacturer's instructions. The absorbance in each well was measured at 520 nm using an ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Mitochondrial Complex I Activity

Rat brains were homogenized with phosphate-buffered saline (PBS) and lysis buffer (N-PER™ obtained from Thermo Scientific™) and centrifuged at 13,000 ×g and 4°C for 15 min. The supernatant was collected and frozen at -80°C prior to analysis. The protein content of each sample was quantified using the Bradford method. Mitochondrial complex I activity was assayed spectrophotometrically at 340 nm using NADH as a substrate[37]. Mitochondrial complex I activity was calculated as NADH oxidized/mg protein.

Statistical Analysis

All the data from *in vivo* studies were collected and analyzed by one-way analysis of variance, followed by Tukey's test. Data were presented as the mean ± SEM (n = 6 experiments). $p < 0.05$ was considered to indicate statistical significance.

Results

The Effect of CMF and MET on IL-1α Expression

ELISA analysis revealed that there were no significant differences in the IL-1α expression levels

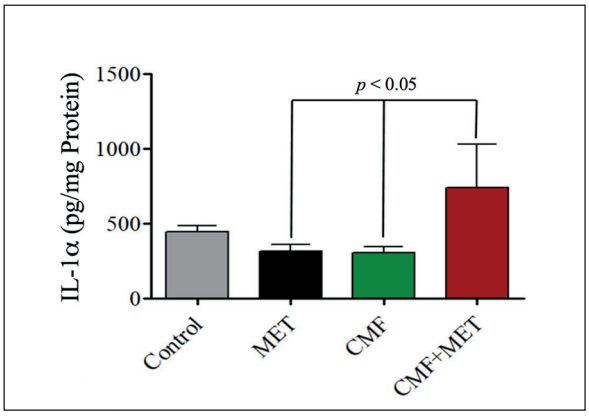

Figure 1. Effect of CMF and MET treatments on IL-1a regulation in rat brains. ELISA analysis showed that IL-a expression levels were significantly higher in the CMF+MET-treated group than in the CMF- and MET-treated groups. However, the IL-1a expression levels the other groups did not differ compared to the levels of the control group. Data analysis was performed using "Tukey multiple comparison test". Statistical significance was accepted at $*p < 0.05$.

of all the treated groups (MET, CMF, and CMF+MET) compared to that in the control group; however, the IL-1α expression level in the CMF+MET group was significantly higher than those in the MET and CMF groups (Figure 1).

The Effect of CMF and MET on IRS-1 Expression

ELISA analysis revealed that there were no significant differences in the IRS-1 expression levels of all the treated groups (MET, CMF, and CMF+MET) compared to that in the control group (Figure 2).

The Effect of CMF and MET on AKT-a Expression

ELISA analysis revealed that there were no significant differences in the AKT-a expression levels of all the treated groups (MET, CMF and CMF+MET) compared to that in the control group (Figure 3).

The effect of CMF and MET on TNF-a expression

ELISA analysis revealed that there were no significant differences in the TNF-a expression levels of all the treated groups (MET, CMF and CMF+MET) compared to that in the control group (Figure 4).

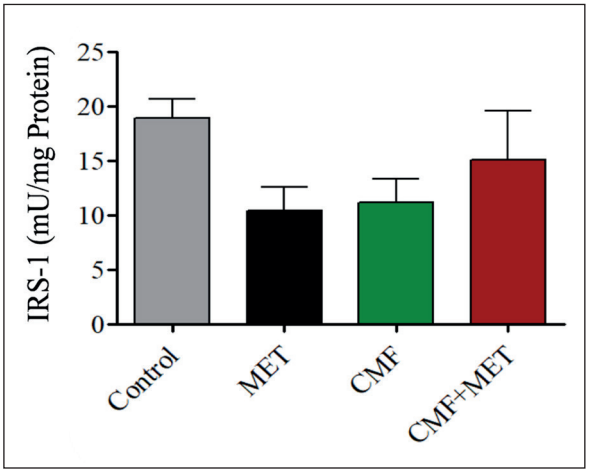

Figure 2. Effect of CMF and MET treatments on IRS-1 regulation in rat brains. ELISA analysis showed that IRS-1 expression levels were slightly reduced in the MET- and CMF-treated groups compared to that in the control group. However, the IRS-1 expression levels in the CMF+MET-treated group did not differ compared to the levels in the control group and were higher than those in the MET- and CMF-treated groups.

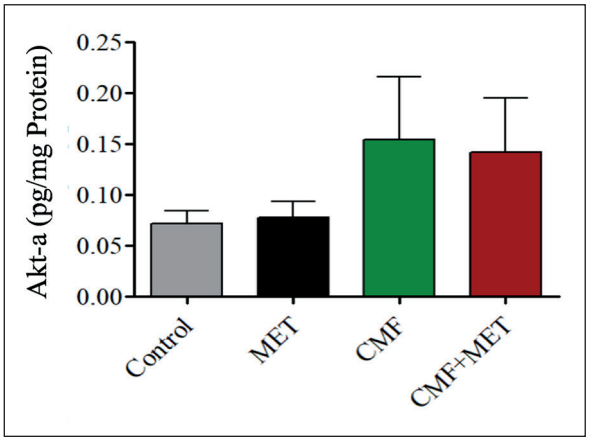

Figure 3. Effect of CMF and MET treatments on regulation of Akt-a regulation in rat brains. ELISA analysis revealed that there were no significant differences in the Akt-a expression levels among the treated groups. However, the CMF- and CMF+MET- treated groups showed slightly higher levels of Akt-a expression compared to those in the MET-alone and control groups.

Mitochondrial Complex I Activity

Analysis of mitochondrial complex I activity confirmed that there were no significant differences in the activity of all the treated groups (MET, CMF and CMF+MET) compared to that in the control group (Figure 5).

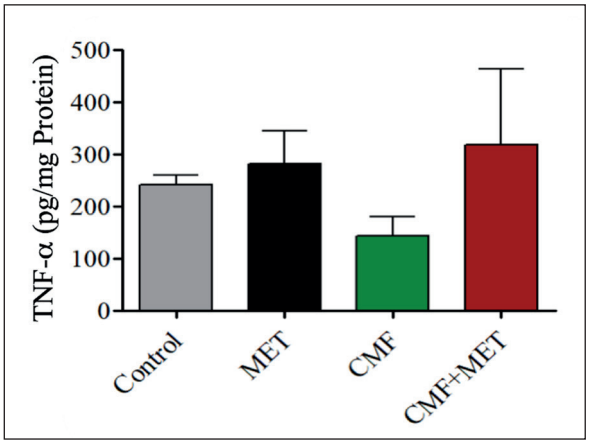

Figure 4. Effect of CMF and MET treatments on regulation of TNF-a regulation in rat brains. ELISA analysis revealed that there were no significant differences in the levels of TNF-a expression levels between the treated groups. However, the CMF-treated groups showed slightly reduced TNF-a expression compered to all the other groups including the control group.

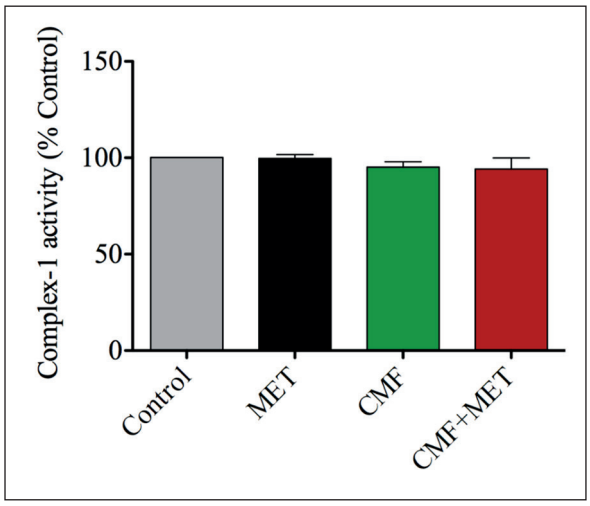

Figure 5. The effect of CMF and MET on mitochondrial complex 1 activity. CMF and MET had no significant effect on mitochondrial complex 1 activity. Protein levels were normalized to the total protein content and presented as a percentage of the levels in the control group, which was set as 100%.

Discussion

In this study, we investigated the mechanism underlying CMF-induced memory impairment. Male rats were used to establish a model of chemo-brain by using CMF to induce cognitive impairment. We then tested the hypothesis that MET protects against CMF-induced memory impairment. A previous study using behavioral tests in a rat model of chemo-brain showed that oral co-administration of MET for two weeks along with two doses of intraperitoneally injected CMF did not prevent the adverse effects of CMF[19]. In a previous study, we showed that metformin induced cognitive impairment and neuroinflammation in CMF-treated rats by conducting hippocampal-dependent behavioral tests including (Y-maze, novel object recognition and elevated-plus maze tests) [Alhowail AH, Almogbel Y, Abdellatif AAH, Aldubayan MA, Alfheeaid HA, Felemban SG, Chigurupati S, Alharbi IF, Alharbi HS. Metformin Induced Cognitive Impairment and Neuroinflammation in CMF-Treated Rats. Int J Pharmacol 2021 (in press)]. In the present study, we showed that CMF and MET treatment can modulate learning and memory processes in the brain. ELISA analysis of proinflammatory cytokine levels in the brain of model rats revealed that IL-1α levels were significantly increased in the CMF+MET group compared with those in the CMF and MET groups. In contrast, CMF and MET treatment did not change IRS-1, TNF-a and Akt-a expression levels or mitochondrial complex 1 activity in the brain. By combining these results with those of our previous behavioral analyses, we concluded that memory is impaired treatment with CMF and MET as a result of alteration of cytokine levels in the brain.

Cytokines are glycoproteins produced by a variety of different cell types and modulate immunological and inflammatory responses as well as playing a crucial role in modulating learning and memory[38]. Elevation of cytokine levels is involved in the pathogenesis of many diseases and disorders associated with cognitive impairment, such as Alzheimer's disease (AD), mild cognitive impairment, and Huntington's disease[39-41]. Recent studies indicate that altered levels of proinflammatory cytokines are associated with cognitive impairment in cancer patients receiving chemotherapeutic agents[42,43]. Thus, the results of our study support the hypothesis that inflammation has a role in the pathogenesis of cognitive problems associated with patients receiving chemotherapy.

The IL-1 family of cytokines affects almost all cells and organs[44] and is involved in the development of autoimmune, inflammatory, and degenerative diseases[45]. IL-1α is a unique member of the IL-1 family in that it is expressed in a healthy state and rarely associated with disease conditions. IL-1α activity is controlled by two types of IL-1 receptors (IL-1R1 and IL-1R2) although the cytokine binds with greater affinity to IL-1R1[46,47]. During cell necrosis, IL-1α is released into the extracellular environment, where it facilitates sterile inflammatory signaling[47]. The cytotoxic effect produced by chemotherapy is thought to be a result of increased IL-1α release from dead cells[48]. It has been reported that patients with AD have elevated serum IL-1α levels compared to control individuals, suggesting its involvement in the pathogenesis[49-51]. The results of the current study suggest that cognitive function was impaired by in the CMF+MET treated group as a result of increased expression of IL-1α compared to the levels in the other treated groups.

Insulin plays an important role in the regulation of memory and other cognitive functions via the IRS-1/PI3k/AKT signaling pathway in the brain[52]. Insulin action is initiated by binding to the insulin receptor and stimulation of tyrosine phosphorylation, followed by the recruitment of IRS-1 pro-

tein[53]. IRS-1 is a widely distributed intracellular signaling adaptor protein that is involved in to the metabolic functions of insulin[52]. IRS-1 stimulates PI3K, which contributes to the phosphorylation of Akt, a serine/threonine-protein kinase. Once Akt is activated, it regulates cell proliferation, survival, metabolism, and glucose transport by inducing the passage of the glucose transporter 4 (GLUT4) isoform across the plasma membrane[54]. Subsequently, activated Akt inhibits glycogen synthase kinase-3 beta (GSK-3β) activity by catalyzing its phosphorylation at Ser9, thereby promoting neural survival[55]. Therefore, downregulation of insulin signaling may lead to decreased glucose metabolism and increased phosphorylation through GSK-3 activation[56]. Liu et al[57] suggests that GSK-3β activation is associated with the memory deficits that occur in advanced age and AD. MET is an antidiabetic drug that lowers blood sugar levels by improving the response to insulin. MET promotes improvement in insulin sensitivity by a complex mechanism that involves processes such as increased insulin receptor tyrosine kinase activity, enhanced glycogen production, upregulated expression of GLUT4 transporters, and increased lipogenesis. Therefore, the improved glycemia associated with MET is not associated with increased circulating levels of insulin.

Although several systemic inflammatory cytokines have been implicated in the pathogenesis of cognitive impairment, the proinflammatory cytokine TNF-a has a significant role. Studies have been shown that an elevated TNF-a levels are associated with rapid development of cognitive dysfunction over a period of six months in AD patients. TNF-a is also associated with exacerbation of behavioral symptoms such as agitation, anxiety, and depression[58]. Elevation in TNF-a levels were shown to be associated with decreased hippocampal volumes and verbal memory impairment in breast cancer survivors [59]. However, in the current study, we demonstrated that concomitant administration of CMF and MET did not change the expression of IRS-1, Akt-a, and TNF-a levels in the brain.

Mitochondria are organelles found in most eukaryotic organisms and play an important role in energy production, calcium regulation, cell metabolism, and synaptic transmission[60]. The energy produced by the mitochondria is stored in form of the small molecule adenosine triphosphate (ATP). Mitochondrial dysfunction is a known cause of cognitive impairment[61]. In addition, it has been reported that mitochondrial dysfunction is induced by some chemotherapeutic agents, such as CYP and MTX[62,63]. In the present study, we established a rat model of chemo-brain using CMF and MET was used to protect against CMF toxicity. Although MET was reported to induce mitochondrial dysfunction, it is also reported to rescue mitochondrial dysfunction caused by diabetes and heart failure. In the present study, there was no significant change in mitochondrial complex I activity in the brain of rats following co-administration of MET and CMF. However, we did not observe significant alterations in mitochondrial complex I activity after two doses of CMF, possibly due to the low dose administered.

Conclusions

In this study, we investigated the levels of proinflammatory cytokines and their relationship with cognitive dysfunction in rats treated with CMF and MET. Our results indicated that treatment with CMF and MET induces cognitive impairment through upregulation of IL-1α expression levels in the brain. Further research is needed to fully elucidate the mechanism responsible for chemotherapy-induced cognitive decline and provide strategies for its prevention and treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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