Elucidating the mechanism underlying cognitive dysfunction by investigating the effects of CMF and MET treatment on hippocampal neurons

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Abstract. – OBJECTIVE: Chemotherapy causes long-term cognitive impairment in cancer survivors. A combination of cyclophosphamide (CYP), methotrexate (MTX), and 5-fluorouracil (5-FU) (i.e., CMF) is widely used for cancer treatment. Metformin (MET), an oral antidiabetic drug, confers protection against the adverse effects of chemotherapeutic agents, such as CYP. To elucidate the potential mechanism underlying cognitive dysfunction, we investigated the impact of CMF and MET treatment on the activities of mitochondrial respiratory chain complexes I and IV, as well as lipid peroxidation, in hippocampal neurons.

MATERIALS AND METHODS: Hippocampal neurons (H19-7) cells were treated for 24 h with MET (0.5 mM) alone; CYP (1 µM), MTX (0.5 µM), and 5-FU (1 µM); and MET (0.5 mM) + CYP (1 µM), MTX (0.5 mM), and 5-FU (1 µM). A 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay was performed to evaluate cell survival. Neurons were collected and homogenized in a neuronal lysis buffer to assess mitochondrial complexes (I and IV) activity and lipid peroxidation.

RESULTS: Compared to the control, MET-treated cells showed no significant difference in survival rate; however, CMF- and CMF + MET-treated cells showed a significant reduction in survival rate. In addition, relative to the control, CMF- and CMF + MET-treated cells showed a reduction in mitochondrial complex I activity, whereas no significant changes were observed in mitochondrial complex IV activity. MET-treated cells showed no significant differences in lipid peroxidation, but CMF- and CMF + MET-treated cells showed a slight increase in lipid peroxidation.

CONCLUSIONS: The reduction in the activity of mitochondrial complex I and a slight increase in lipid peroxidation levels may explain the cognitive impairment following CMF and MET treatments.

Key Words: Chemotherapy, Cyclophosphamide, Methotrexate, 5-Fluorouracil, Metformin, Hippocampal neurons, Mitochondrial function.

Introduction

Chemotherapy is one of the principal modes of cancer treatment that is effective against several types of tumors; however, it also affects normal tissues due to its lack of selectivity. Moreover, chemotherapy can induce oxidative stress and inflammation, causing toxicity and various adverse effects. Chemobrain or chemofog is an important but less investigated side effect characterized by deficits in concentration, memory, decision-making, learning, and language during chemotherapy and after its cessation. As many as 70% of cancer survivors show signs of chemobrain; in 35% of cases, these signs can persist for up to 5 years. Hippocampal neurons regulate mainly cognitive function and memory forma-
tion. Therefore, alterations in hippocampal neuron proteins expression levels or functions can impair memory. To date, the precise etiology and mechanisms underlying chemobrain remain poorly understood.

The combination of cyclophosphamide (CYP), methotrexate (MTX), and 5-fluorouracil (5-FU) (CMF) is commonly used for breast cancer treatment. CYP, a multifunctional alkylating agent, exerts its effects through DNA alkylation, consequently blocking DNA transcription and RNA translation. MTX is a folate antagonist that inhibits several nucleotide synthesis-associated enzymes such as dihydrololate reductase, catalyzing the reduction of dihydrololate to tetrahydrofols. 5-FU is an antimitabolite of the naturally occurring nucleobase uracil that inhibits thymidylate synthase to prevent DNA and RNA synthesis. CMF can cross the blood-brain barrier, conferring protection against cytotoxic agents, including chemotherapeutic drugs. Koppelmans et al. investigated neuropsychological performance in breast cancer survivors more than 20 years after adjuvant CMF chemotherapy. They reported that they performed worse on average than random population controls on neuropsychological tests. In addition, CMF administration has been reported to decrease neurogenesis in the hippocampus in rodent models of chemobrain, leading to cognitive function impairment.

Metformin (MET) is an oral antidiabetic drug frequently used as first-line treatment for type II diabetes mellitus. It is also used to treat other conditions, such as polycystic ovary syndrome, obesity, and metabolic syndrome. MET acts by activating the adenosine monophosphate-activated protein kinase (AMPK) pathway that regulates other cell signaling pathways. AMPK activates anti-inflammatory and anti-cancer effects. AMPK inhibits the mammalian target of rapamycin that plays a key role in cell growth. AMPK activates the phosphatidylinositol 3-kinase/protein kinase B (Akt) signaling pathway that plays a critical role in glucose transporter trafficking to the cell surface. MET treatment reduces the risk of cancer development in patients, acting synergistically with several chemotherapeutic drugs to inhibit tumor cell growth. MET also rescues MTX-induced memory impairment in a rat model. In addition, MET lowers the risk of cognitive impairment in patients with diabetes via AMPK-dependent and AMPK-independent mechanisms.

Our previous studies reported that CMF and MET treatments induced cognitive impairment by modulating IL-6 and IL-6α levels in rat models of chemobrain. This was assessed using hippocampal-dependent tasks such as Y-maze, novel object recognition, and elevated plus maze. Metformin induced cognitive impairment and neuroinflammation in CMF-treated rats. In this study, we further investigated the effects of CMF and MET treatment on cognitive function by assessing mitochondrial respiratory activities of chain complexes I and IV as well as lipid peroxidation in H19-7 hippocampal neuron cells.

## Materials and Methods

### Chemicals

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

### H19-7 Hippocampal Neurons

Rat embryonic hippocampal neuronal (H19-7/IGF-IR) cells were purchased from ATCC (Manassas, VA, USA) and grown in poly L-lysine-coated 6-well plates in DMEM supplemented with 10% fetal bovine serum, 200 µg/mL G-418, and 1 µg/mL puromycin at 34°C, as previously described. The cells were exposed to three different treatments for 24 h: MET (0.5 mM) alone; CYP (1 µM), MTX (0.5 µM), and 5-FU (1 µM); MET (0.5 mM) plus CYP (1 µM), MTX (0.5 mM), and 5-FU (1 µM). Neuron cells were spread in a 75-cm² poly-L-lysine-coated tissue culture flask and harvested by trypsinization (trypsin 0.25% [w/v] EDTA) after the cells reached approximately 75% confluency (2–3 days).

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Reduction Assay

Neuronal survival was determined using the colorimetric MTT assay. Hippocampal (H19-7) cells were plated in 48-well culture plates at a
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density of 2,000 cells per well and cultured for 24 h. The growth medium was then replaced with a medium with or without therapeutic agents. After incubation for 24 h, the medium was replaced by 1 mg/mL MTT, followed by incubation for 4 h at 34°C and 5% CO₂. Subsequently, the cell suspension, including the supernatant, was removed by suction, and the formazan crystals were solubilized with DMSO. Absorbance was measured at 570 nm using a microplate reader².

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Hippocampal neuron cells were lysed using N-PER™ neuronal protein extraction reagent (Thermo Scientific™, Paisley, UK) and sonicated with a Qsonica homogenizer (Newtown, CT, USA) at a frequency of 30 Hz pulses for 20 s. After centrifugation at 12,000 × g for 10 min, the supernatant was collected, and 200-µL aliquots were stored at -80°C. The protein content of each sample was quantified using the bicinecinonic acid (BCA) assay (Pierce), and mitochondrial complex IV activity was analyzed using an ELISA kit (MyBioSource Inc., San Diego, CA, USA) according to the manufacturer’s instructions. The absorbance of each well was measured at 520 nm using an ELX800 microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

**Mitochondrial Complex I Activity**

Hippocampal neurons were homogenized in PBS and lysis buffer (N-PER™) and centrifuged at 12,000 × g at 4°C for 10 min. The supernatant was collected and stored at -80°C before analyses. The protein content in each sample was quantified using the Bradford method. Mitochondrial complex I activity was spectrophotometrically assayed at 340 nm using NADH as the substrate³ and was calculated as NADH oxidized/mg protein.

**Lipid Peroxidation Assay**

Rat embryonic hippocampal neuron cells were cultured and exposed to three different treatments for 24 h: MET (0.5 mM) alone, CYP (1 µM), MTX (0.5 µM), 5-FU (1 µM), MET (0.5 mM) plus CYP (1 µM), MTX (0.5 mM), and 5-FU (1 µM). Cells were collected and homogenized in lysis buffer (N-PER™), followed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant obtained was transferred to a new centrifuge tube. The total protein content of all samples was estimated using the bicinecinonic acid assay before performing the lipid peroxidation assay. Lipid peroxidation levels were assessed using a spectrophotometric method with thiobarbituric acid. The lipid peroxidation index was estimated based on the formation of thiobarbituric acid-reactive substances (TBARS) at 532 nm. TBARS was normalized to total protein content as TBARS formed/mg protein²,³².

**Statistical Analysis**

Data from the in vitro studies were collected and analyzed using One-way analysis of variance, followed by Tukey’s test. Values represent the mean ± SEM (n = 5 experiments). Statistical significance was set at p < 0.05.

**Results**

**Effects of CMF and MET on Cell Survival**

The cells were treated for 24 h with MET, CMF, or CMF + MET, as described earlier, to assess the effects of MET and CMF treatment on hippocampal neuronal cell survival. Treatment with MET (0.5 mM) did not significantly affect cell survival rate, whereas treatment with CMF and CMF+MET reduced the cell survival rate by 20% and 20%, respectively (Figure 1).

**Effects of CMF and MET on Mitochondrial Complex I Activity**

Compared with the control, MET-treated cells showed no significant difference, but CMF- and

![Figure 1. MTT assay data showing the survival rate of hippocampal neurons (H19-7) treated with MET, CMF, and CMF+MET. MET-treated neuronal cells showed no significant changes in survival rate, while CMF- and CMF+MET-treated cells showed a significant reduction in survival rate. Data analysis was performed using Tukey multiple comparison test. *p < 0.05.](image)
CMF+MET-treated cells showed a significant difference in mitochondrial complex I activity (Figure 2).

**Effects of CMF and MET on Mitochondrial Complex IV Activity**

Compared with the control, MET-, CMF-, and CMF+MET-treated cells showed no significant differences in mitochondrial complex IV activity (Figure 3).

**Effects of CMF and MET on Lipid Peroxidation**

Compared with the control, MET-treated cells showed no significant differences in lipid peroxidation; however, CMF- and CMF+MET-treated cells showed a slight increase in lipid peroxidation (Figure 4).

**Discussion**

In this study, we investigated the effects of CMF treatment on mitochondrial function and lipid peroxidation that are potentially associated with chemobrain. Hippocampal neurons play a central role in memory formation and induce cognitive impairment in cancer-treatment survivors. MET has been hypothesized to confer protection against CMF-induced memory impairments. In our previous study involving behavioral tests and biochemical analyses in a rat model of chemobrain, we observed that oral co-administration of MET for 2 weeks along with two doses of intraperitoneally injected CMF did not prevent the adverse effects of CMF. Herein, we identified that CMF and MET treatment modulated the mitochondrial respiratory chain complexes in hippocampal neurons. MTT assay results revealed that CMF treatment reduced the cell survival rate by 20%, but MET treatment did not; this could be one of the reasons for memory impairment.

Mitochondria, a hallmark of eukaryotic cells, is involved in energy supply and calcium regulation, cell metabolism, and synaptic plasticity. The energy generated by the mitochondria is stored in the form of ATP. Mitochondrial dysfunction has been associated with cognitive defects resulting from neural stem cell depletion and impaired

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**Figure 2.** Relative to the control, CMF- and CMF+MET-treated cells showed reduced mitochondrial complex I activity. Data analysis was performed using Tukey multiple comparison test. *p < 0.05.

**Figure 3.** Relative to the control, no significant differences in mitochondrial complex IV activity were observed on treatment with MET, CMF, or CMF + MET. Data analysis was performed using Tukey multiple comparison test. *p < 0.05.

**Figure 4.** Relative to the control, MET-, CMF-, and CMF+MET-treated cells did not show a significant difference in lipid peroxidation. Data analysis was performed using Tukey multiple comparison test. *p < 0.05.
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neurogenesis\textsuperscript{35}. In addition, CYP and MTX treatments have been reported to lead to deficits in mitochondrial function\textsuperscript{36,37}. In the current study, we evaluated the activity of mitochondrial respiratory chain complexes I and IV and the protective effects of MET against CMF toxicity. Although MET has been reported to induce mitochondrial dysfunction when used alone\textsuperscript{38}, it has also been reported to prevent mitochondrial dysfunction caused by diabetes and heart failure\textsuperscript{39}. In response to CMF treatment, hippocampal neurons showed a significant change in mitochondrial respiratory chain complex I activity. However, no significant alterations in mitochondrial complex I activity were observed after administration of the two doses of CMF, possibly because the dose was low.

Excessive lipid peroxidation is an indicator of oxidative stress. It can induce neurotoxicity\textsuperscript{39}; excessive lipid oxidation can alter the physical properties of cellular membranes and induce covalent modification of proteins and nucleic acids\textsuperscript{40}. In CMF-treated cells, the level of TBARS, a by-product of lipid peroxidation, was slightly increased, indicating the occurrence of oxidative stress. Oxidative stress generates electrophilic aldehydes that can slow down the cell cycle and cause cellular arrest\textsuperscript{41}. Furthermore, oxidative stress can occur upon exposure to chemotherapeutic agents, including CMFs. Therefore, it can be hypothesized that cognitive impairment following CMF therapy results from neuronal oxidative stress. Several lines of evidence\textsuperscript{42} illustrated the effects of mitochondrial dysfunction on cognitive impairment. It seems that CYP inhibits DNA transcription, potentially causing cellular energy deficits in neurons, and ultimately inducing cognitive impairment. In this study, we investigated the effects of CMF and MET treatments on the activity of mitochondrial complexes I and IV. Neither CMF- nor CMF + MET-treated cells showed any impairment in mitochondrial complex I activity; similarly, CMF- and MET-treated cells did not show any significant changes in mitochondrial complex IV activity.

Conclusions

Herein, we aimed at elucidating the potential mechanism underlying cognitive dysfunction by investigating the effects of CMF and MET treatment on mitochondrial respiratory chain complex I and IV activities, as well as lipid peroxidation in H19-7 hippocampal neurons. We found that CMF and MET treatment significantly reduced the activity of mitochondrial complex I and slightly increased the level of lipid peroxidation. We believe that these changes explain cognitive impairment following CMF and MET treatment. Further studies are warranted to elucidate the mechanisms underlying chemotherapy-induced cognitive impairment comprehensively. The data reported in such studies should facilitate the development of effective strategies to prevent and treat chemobrain.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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