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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[1,2]. 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[3]. As many as 70% of cancer survivors show signs of chemobrain; in 35% of cases, these signs can persist for up to 5 years[4]. Hippocampal neurons regulate mainly cognitive function and memory forma-

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tion. Therefore, alterations in hippocampal neuron proteins expression levels or functions can impair memory[5]. 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[6,7]. CYP, a multifunctional alkylating agent, exerts its effects through DNA alkylation, consequently blocking DNA transcription and RNA translation[8]. MTX is a folate antagonist that inhibits several nucleotide synthesis-associated enzymes such as dihydrofolate reductase, catalyzing the reduction of dihydrofolate to tetrahydrofolate[1,9]. 5-FU is an antimetabolite of the naturally occurring nucleobase uracil that inhibits thymidylate synthase to prevent DNA and RNA synthesis[10]. CMF can cross the blood-brain barrier, conferring protection against cytotoxic agents, including chemotherapeutic drugs[11-13]. Koppelmans et al[14] 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[14]. In addition, CMF administration has been reported to decrease neurogenesis in the hippocampus in rodent models of chemobrain, leading to cognitive function impairment[13,15].

Metformin (MET) is an oral antidiabetic drug frequently used as first-line treatment for type II diabetes mellitus[16]. It is also used to treat other conditions, such as polycystic ovary syndrome, obesity, and metabolic syndrome[17]. MET acts by activating the adenosine monophosphate-activated protein kinase (AMPK) pathway that regulates other cell signaling pathways[18]. AMPK activates anti-inflammatory and anti-cancer effects[16,17]. AMPK inhibits the mammalian target of rapamycin that plays a key role in cell growth[19]. AMPK activates the phosphatidylinositol 3-kinase/protein kinase B (Akt) signaling pathway[20] that plays a critical role in glucose transporter trafficking to the cell surface[21]. MET treatment reduces the risk of cancer development in patients[22], acting synergistically with several chemotherapeutic drugs to inhibit tumor cell growth[23-25]. MET also rescues MTX-induced memory impairment in a rat model[26]. In addition, MET lowers the risk of cognitive impairment in patients with diabetes via AMPK-dependent and AMPK-independent mechanisms[27-29]. Our previous studies[30] 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[31]. 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 (Utoral®) 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[20]. 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-cm^2 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). The cells were then plated into poly L-lysine-coated 6-well plates at a density of 10,000 cells per well. The cells were then supplemented with 5% CO_2, incubated at 34°C, and cultured for ten passages. Therapeutic agents were added to the medium when the cells reached approximately 70% confluence (about 24 h) before extraction.

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

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% CO_2. 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 reader[2].

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 bicinchoninic 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 absorbance 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 substrate[31] 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 bicinchoninic 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 protein[2,32].

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

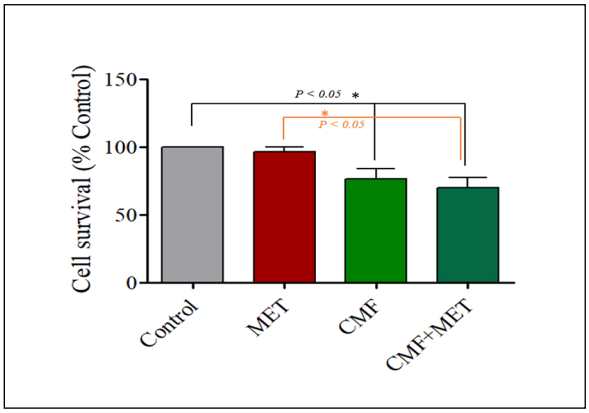

Figure 1. MTT assay data showing the survival rate of hippocampal neurons (H19-7) treated with MET, CMF, and CMF+MET. MET-treated neuronal cells showed no significant changes in survival rate, while CMF- and CMF+MET-treated cells showed a significant reduction in survival rate. Data analysis was performed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

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).

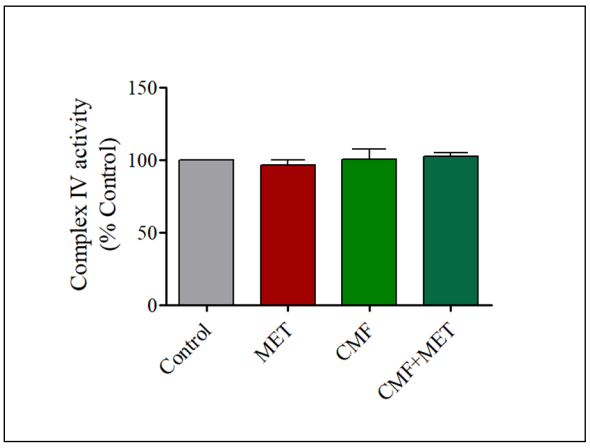

Figure 3. Relative to the control, no significant differences in mitochondrial complex IV activity were observed on treatment with MET, CMF, or CMF + MET. Data analysis was performed using Tukey multiple comparison test. *$p < 0.05$.

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[15,31]. 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[33,34]. The energy generated by the mitochondria is stored in the form of ATP[34]. Mitochondrial dysfunction has been associated with cognitive defects resulting from neural stem cell depletion and impaired

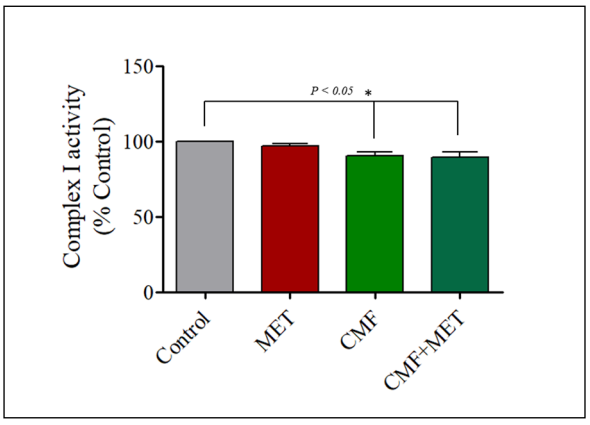

Figure 2. Relative to the control, CMF- and CMF+MET-treated cells showed reduced mitochondrial complex I activity. Data analysis was performed using Tukey multiple comparison test. *$p < 0.05$.

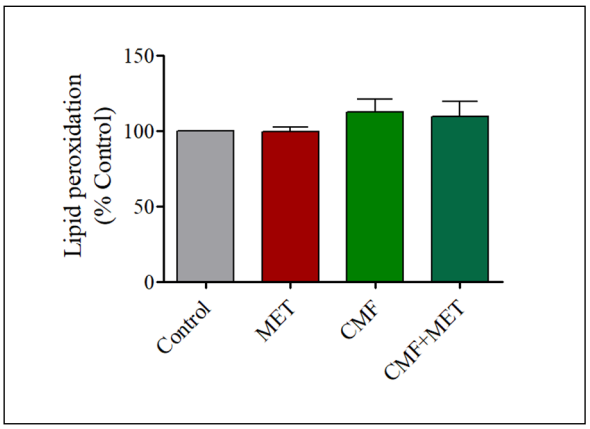

Figure 4. Relative to the control, MET-, CMF-, and CMF+MET-treated cells did not show a significant difference in lipid peroxidation. Data analysis was performed using Tukey multiple comparison test. *$p < 0.05$.

neurogenesis[35]. In addition, CYP and MTX treatments have been reported to lead to deficits in mitochondrial function[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[38], it has also been reported to prevent mitochondrial dysfunction caused by diabetes and heart failure[38]. 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[39]; excessive lipid oxidation can alter the physical properties of cellular membranes and induce covalent modification of proteins and nucleic acids[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[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[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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References

1) Felemban SG, Aldubayan MA, Alhowail AH, Almami IS. Vitamin B17 ameliorates methotrexate-induced reproductive toxicity, oxidative stress, and testicular injury in male rats. Oxid Med Cell Longev 2020; 2020: 4372719.
2) Alhowail AH, Bloemer J, Majrashi M, Pinky PD, Bhattacharya S, Yongli Z, Bhattacharya D, Eggert M, Woodie L, Buabeid MA, Johnson N, Broadwater A, Smith B, Dhanasekaran M, Arnold RD, Suppiramaniam V. Doxorubicin-induced neurotoxicity is associated with acute alterations in synaptic plasticity, apoptosis, and lipid peroxidation. Toxicol Mech Methods 2019; 29: 457-466.
3) Argyriou AA, Assimakopoulos K, Iconomou G, Giannakopoulou F, Kalofonos HP. Either called "chemobrain" or "chemofog," the long-term chemotherapy-induced cognitive decline in cancer survivors is real. J Pain Symptom Manage 2011; 41: 126-139.
4) Turrina S, Gibelli F, De Leo D. Chemotherapy-induced cognitive impairment from the forensic medicine perspective: A review of the updated literature. J Forensic Leg Med 2020; 76: 102070.
5) Tsai YW, Yang YR, Sun SH, Liang KC, Wang RY. Post ischemia intermittent hypoxia induces hippocampal neurogenesis and synaptic alterations and alleviates long-term memory impairment. J Cereb Blood Flow Metab 2013; 33: 764-773.
6) Clarke M, Coates A, Darby S, Davies C, Gelber R, Godwin J, Goldhirsch A, Gray R, Peto

R, Pritchard K, Wood W. Adjuvant chemotherapy in oestrogen-receptor-poor breast cancer: patient-level meta-analysis of randomised trials. Lancet 2008; 371: 29-40.

7) 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
8) Iqubal A, Iqubal MK, Sharma S, Ansari MA, Najmi AK, Ali SM, Ali J, Haque SE. Molecular mechanism involved in cyclophosphamide-induced cardiotoxicity: Old drug with a new vision. Life Sci 2019; 218: 112-131.
9) Verberne EA, de Haan E, van Tintelen JP, Lindhout D, van Haelst MM. Fetal methotrexate syndrome: A systematic review of case reports. Reprod Toxicol 2019; 87: 125-139.
10) Wang N, Yang L, Dai J, Wu Y, Zhang R, Jia X, Liu C. 5-FU inhibits migration and invasion of CRC cells through PI3K/AKT pathway regulated by MARCH1. Cell Biol Int 2020; 45: 368-381.
11) Upadhyay RK. Drug delivery systems, CNS protection, and the blood brain barrier. BioMed Res Int 2014; 2014: 1-37.
12) Anderson JE, Trujillo M, McElroy T, Groves T, Alexander T, Kiffer F, Allen AR. Early effects of cyclophosphamide, methotrexate, and 5-fluorouracil on neuronal morphology and hippocampal-dependent behavior in a murine model. Toxicol Sci 2019; 173 : 156-170.
13) Briones TL, Woods J. Dysregulation in myelination mediated by persistent neuroinflammation: Possible mechanisms in chemotherapy-related cognitive impairment. Brain, Behav Immun 2014; 35: 23-32.
14) Koppelmans V, Breteler MMB, Boogerd W, Seynaeve C, Gundy C, Schagen SB. Neuropsychological performance in survivors of breast cancer more than 20 years after adjuvant chemotherapy. J Clin Oncol 2012; 30: 1080-1086.
15) Briones TL, Woods J. Chemotherapy-induced cognitive impairment is associated with decreases in cell proliferation and histone modifications. BMC Neurosci 2011; 12: 124.
16) Foretz M, Guigas B, Viollet B. Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus. Nat Rev Endocrinol 2019; 15: 569-589.
17) Rêgo DF, Pavan LMC, Elias ST, De Luca Canto G, Guerra ENS. Effects of metformin on head and neck cancer: A systematic review. Oral Oncol 2015; 51: 416-422.
18) Agius L, Ford BE, Chachra SS. The Metformin Mechanism on Gluconeogenesis and AMPK Activation: The Metabolite Perspective. Int J Mol Sci 2020; 21: 3240.
19) Ling NXY, Kaczmarek A, Hoque A, Davie E, Ngoei KRW, Morrison KR, Smiles WJ, Forte GM, Wang T, Lie S, Dite TA, Langendorf CG, Scott JW, Oakhill JS, Petersen J. mTORC1 directly inhibits AMPK to promote cell proliferation under nutrient stress. Nat Metab 2020; 2: 41-49.
20) Tao R, Gong J, Luo X, Zang M, Guo W, Wen R, Luo Z. AMPK exerts dual regulatory effects on the PI3K pathway. J Mol Signal 2010; 5: 1
21) Alharbi I, Alharbi H, Almogbel Y, Alalwan A, Alhowail A. Effect of metformin on doxorubicin-induced memory dysfunction. Brain Sci 2020; 10: 3
22) Saraei P, Asadi I, Kakar MA, Moradi-Kor N. The beneficial effects of metformin on cancer prevention and therapy: a comprehensive review of recent advances. Cancer Manag Res 2019; 11: 3295-3313.
23) Peng M, Darko KO, Tao T, Huang Y, Su Q, He C, Yin T, Liu Z, Yang X. Combination of metformin with chemotherapeutic drugs via different molecular mechanisms. Cancer Treat Rev 2017; 54: 24-33.
24) Soo JSS, Ng CH, Tan SH, Malik RA, Teh YC, Tan BS, Ho GF, See MH, Taib NAM, Yip CH, Chung FFL, Hii LW, Teo SH, Leong CO. Metformin synergizes 5-fluorouracil, epirubicin, and cyclophosphamide (FEC) combination therapy through impairing intracellular ATP production and DNA repair in breast cancer stem cells. Apoptosis 2015; 20: 1373-1387.
25) Wen KC, Sung PL, Wu ATH, Chou PC, Lin JH, Huang CYF, Yeung SCJ, Lee MH. Neoadjuvant metformin added to conventional chemotherapy synergizes anti-proliferative effects in ovarian cancer. J Ovarian Res 2020; 13: 1
26) Sritawan N, Prajit R, Chaisawang P, Sirichoat A, Pannangrong W, Wigmore P, Welbat JU. Metformin alleviates memory and hippocampal neurogenesis decline induced by methotrexate chemotherapy in a rat model. Biomed Pharmacother 2020; 131: 110651.
27) Ng TP, Feng L, Yap KB, Lee TS, Tan CH, Winblad B. Long-term metformin usage and cognitive function among older adults with diabetes. J Alzheimer's Dis 2014; 41: 61-68.
28) Urruticoechea A, Alemany R, Balart J, Villanueva A, Vinals F, Capella G. Recent advances in cancer therapy: an overview. Curr Pharm Des 2010; 16: 3-10.
29) Zhang QQ, Li WS, Liu Z, Zhang HL, Ba YG, Zhang RX. Metformin therapy and cognitive dysfunction in patients with type 2 diabetes. Medicine 2020; 99: 19378.
30) Alhowail AH, Almogbel YS, Abdellatif AAH, Alsalehi NF, Alghenaim FA, Aldubayan MA, Felemban SG. CMF and MET treatment induce cognitive impairment through upregulation of IL-1alpha in rat brain. Eur Rev Med Pharmacol Sci 2021; 25: 4385-4393.
31) Thrash-Williams B, Karuppagounder SS, Bhattacharya D, Ahuja M, Suppiramaniam V, Dhanasekaran M. Methamphetamine-induced dopaminer-

gic toxicity prevented owing to the neuroprotective effects of salicylic acid. Life Sci 2016; 154: 24-29.

32) Alhowail AH, Almogbel Y, Abdellatif AH, 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 2022; 18: 228-235.

33) Knott AB, Bossy-Wetzel E. Impairing the mitochondrial fission and fusion balance: a new mechanism of neurodegeneration. Ann N Y Acad Sci 2008; 1147: 283-292.

34) Alhowail A. Potential mechanisms of metformin-induced memory impairment. Eur Rev Med Pharmacol Sci 2021; 25: 4757-4761.

35) Khacho M, Clark A, Svoboda DS, MacLaurin JG, Lagace DC, Park DS, Slack RS. Mitochondrial dysfunction underlies cognitive defects as a result of neural stem cell depletion and impaired neurogenesis. Hum Mol Genet 2017; 26: 3327-3341.

36) Chandra D, Crouch ML, Knowels G, Stuppard R, Ericson NG, Bielas JH, Marcinek DJ, Syrjala KL. Cyclophosphamide leads to persistent deficits in physical performance and in vivo mitochondria function in a mouse model of chemotherapy late effects. PLoS One 2017; 12: e0181086.

37) Heidari R, Ahmadi A, Mohammadi H, Ommati MM, Azarpira N, Niknahad H. Mitochondrial dysfunction and oxidative stress are involved in the mechanism of methotrexate-induced renal injury and electrolytes imbalance. Biomed Pharmacother 2018; 107: 834-840.

38) Yang M, Darwish T, Larraufie P, Rimmington D, Cimino I, Goldspink DA, Jenkins B, Koulman A, Brighton CA, Ma M, Lam BYH, Coll AP, O'Rahilly S, Reimann F, Gribble FM. Inhibition of mitochondrial function by metformin increases glucose uptake, glycolysis and GDF-15 release from intestinal cells. Sci Rep 2021; 11: 1.

39) Zeiger SLH, Musiek ES, Zanoni G, Vidari G, Morrow JD, Milne GJ, McLaughlin B. Neurotoxic lipid peroxidation species formed by ischemic stroke increase injury. Free Radic Biol Med 2009; 47: 1422-1431.

40) Gaschler MM, Stockwell BR. Lipid peroxidation in cell death. Biochem Biophys Res Commun 2017; 482: 419-425.

41) Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem 2008; 283: 21837-21841.

42) Scaglia F. The role of mitochondrial dysfunction in psychiatric disease. Dev Dis Res Rev 2010; 16: 136-143.