Endoplasmic reticulum stress plays an important role in methotrexate-related cognitive impairment in adult rats

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Abstract: The patients receiving methotrexate (MTX) treatment are inclined to suffer from cognition impairment, since MTX can induce apoptosis of neurons, while the underlying molecular mechanisms remain unknown. Thus we hypothesized that MTX-induced apoptosis of hippocampal neurons via activating endoplasmic reticulum stress (ERS) pathway, which leads to cognitive impairment in adult rats. In order to confirm our hypothesis, twenty male Sprague-Dawley rats weighting 180-220 g were divided into two groups: the control group (physiological saline) and the MTX60 group (MTX 60 mg/kg). Spatial memory was assayed by the Morris water maze test (MWM). In the mean time, another twenty-four rats were divided into four groups: the control group (physiological saline), MTX 60 (MTX, 60 mg/kg), MTX100 (MTX, 100 mg/kg) and MTX250 (MTX, 250 mg/kg). Then, we observed the pathological changes of the hippocampus by hematoxylin-eosin stained. The expressions of C/EBP homologous protein (CHOP) and caspase-12 in the hippocampus were determined by Western blot and immunofluorescence. The apoptosis of neurons were assessed by TUNEL assay. The Morris water maze test showed that MTX induced spatial memory impairment in adult rats ($P<0.05$). The degenerated or apoptotic neurons were condensed and the number of neurons with nuclear pyknosis increased significantly in hippocampus CA1 area of rats in MTX groups. Additionally, both protein expressions of CHOP and caspase-12 and number of TUNEL positive cells were significantly increased in these MTX groups ($P<0.05$). The present results suggested that ERS mediated by apoptosis of hippocampal neurons might play an important role in the mechanism of MTX-induced cognitive impairment in adult rats.

Keywords: Methotrexate, apoptosis, cognitive impairment, endoplasmic reticulum stress, hippocampal neurons

Introduction

Methotrexate (MTX) is a well-known cytostatic agent used in combination chemotherapy for lymphatic system malignancies. It significantly improves the survival of patients with lymphoma or acute lymphoblastic leukemia (ALL), and effectively prevents the relapse of central nervous system leukemia [1, 2]. However, intensified treatment protocols brought about a parallel increase in neurotoxicity [3]. Particularly, deficits in neurological and cognitive function are distressing complications of therapy for survivors, as these can last for years after treatment and affect learning or occupational performance [4, 5].

Although some literatures had reported that potential mechanisms for cognitive impairment induced by MTX related to the apoptosis of hippocampal neurons [6, 7]. The precise mecha-
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sion of ER-resident proteins including calreticulin, protein disulphide isomerase A3 and A4, and GRP78 in a time-dependent manner [12]. Therefore, we propose that MTX-induced cognitive deficits may be related to neuronal apoptosis and ERS in hippocampus.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighting between 180 g and 220 g were acquired from the Animal Center of the Southern Medical University (Guangzhou, China). The rat experiments were approved by the Animal Use and Protection Committee of Southern Medical University, and performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were individually housed under a 12/12 h light/dark cycle and given food, water with ad libitum fed. The rats were permitted to acclimate to their surroundings for 1 week to become accustomed to the experimenter.

Drug treatment

Twenty of these rats received either MTX (Pfizer, USA) or physiological saline, and MTX was administered via intraperitoneal injection at a dose of 60 mg/kg. Cognitive performance was evaluated shortly on days 1 to 5 after drug treatment with the Morris water maze test.

Another twenty-four rats were used to explore the effects of different doses of MTX on neuronal apoptosis and CHOP and caspase-12 expression levels in hippocampus after administration of an intraperitoneal injection of MTX (60 mg/kg, 100 mg/kg, 250 mg/kg respectively). The control group was injected intraperitoneally with 0.9% saline.

Sample preparation

On the third day after drug treatment, twenty-four rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.35 ml/kg). The rats were perfused via left ventricle with approximately 300 ml normal saline. Then, the rat’s hippocampus was quickly separated on ice. Half of the hippocampus of the rats per group were removed and fixed in 4% paraformaldehyde for over 24 h. After that they were routinely dehydrated, paraffin embedded and sectioned for hematoxylin and eosin (HE) staining, immunohistochemical staining, and TUNEL assays. The other half of hippocampus per group were frozen immediately on dry ice, and stored at -80°C for protein analysis.

Morris water maze test (MWM)

After MTX treatment, the spatial memory abilities of rats were tested with MWM. The maze mainly consisted of a circular swimming pool (200 cm diameter and 60 cm height) which was filled with water to a depth of 30 cm and the water was made opaque by the addition of black non-toxic ink. The water temperature was maintained at 22°C-24°C. The maze was divided into 4 quadrants at equal distance on the rim. An invisible platform (20 cm diameter) was submerged 1.5 cm below the surface of the water and fixed the location in the centre of the second quadrant of the pool.

During the four consecutive days, the rats were tested four times per day to learn the position of the platform. On each trial, the test began by releasing the rat from one of our predetermined starting positions on the rim of the pool. The starting position was varied on each trial in a quasi-random sequence. The rat faced the pool wall and allowed them to swim and find the hidden platform in a maximum of two minutes. Rats who failed to swim to the platform within 120 seconds would be gently guided to the platform. After arriving at the platform, the rat was allowed to remain there for 15 seconds. The latency time to find the hidden platform was recorded and the average time of 4 trials represented as the daily result for the rat. On the 5th day, the hidden platform was removed, and each rat was allowed to swim freely for 60 seconds. The swimming time in the previous platform site was recorded during the probe trial.

Histological examination

The hippocampi were paraffin-embedded, sectioned into 4-μm serial sections, and stained with hematoxylin and eosin (H&E), as previously described [13]. Then the HE-stained sections of the hippocampus were observed with an inverted microscope.

TUNEL assay

A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was
performed to determine the degree of DNA fragmentation using a commercial kit (promega, United States). Briefly, after deparaffinization, the sections were treated with 20 μg/ml proteinase K for 30 min, fixed in 4% paraformaldehyde for 10 min, and balanced with equilibration Buffer for 10 min at room temperature. The sections were incubated with rTdT reaction liquid (contained Equilibration Buffer, Biotinylated Nucleotide Mix, and rTdT Enzyme) for 60 min at 37°C, and washed with 2X SSC solution for 15 min. After treatment with 3% H₂O₂ for 10 min, the sections were washed with PBS for 5 min×3, and incubated with Streptavidin HRP antibody at 37°C for 30 min. The sections were washed with PBS for 15 min at room temperature, followed by hematoxylin counterstaining. Apoptotic nuclei were labelled with DAB staining. The TUNEL positive cells and the total number of cells in the hippocampus were counted under 400× magnification.

**Western blot**

Fresh frozen hippocampi were fully homogenized in RAPI lysis buffer (Beyotime Biotechnology, China) with Phenylmethanesulfonyl fluoride (PMSF). After centrifuging the tissue homogenate at 12,000 r/min for 20 min, separates the supernatant to measure the protein concentration with BCA protein assay kit (Beyotime Biotechnology, China). The samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) for 2 h at room temperature, then they were incubated with primary antibody (CHOP, 1:800, Bioworld Technology, Dublin, USA), caspase-12 (1:1000, Bioworld Technology, Dublin, USA) at 4°C overnight. Goat anti-rabbit IgG were used as secondary antibodies (1:1000, Boster, China) and were coupled with the blots for another 2 h at room temperature. The blot developed with the enhanced Chemiluminescence (ECL; Millipore, Bedford, MA, USA). GADPH (1:10000) (Kang Chen Bio-tech, China) was used as a loading control. The immunoblot signal was visualized with an image analysis system using BIO-ID software (Vilber Lourmat, Marne La Vallec, France) and the integrated optical density of the protein band was determined using Image-J software.

**Immunofluorescence staining**

The mounted sections were washed in 0.01 M PBS, and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min at room temperature. To facilitate antigen retrieval, the sections were heated to boiling at high temperature, using a 0.01 mol/L citrate buffer (pH 6.0) in microwave oven, and continued to boil at low temperature for 8 min. Then the sections were incubated in 10% normal horse serum in PBS for 30 min at 37°C, incubated at 4°C overnight in CHOP antibody (1:100, ImmunoWay Biotechnology, USA) or Caspase-12 antibody (1:100, ImmunoWay Biotechnology, USA) in PBS, washed in PBS (3×5 min), incubated for 30 min at room temperature with horseradish peroxidase (HRP) (ChemMateTM DAKO EnVisionTM Detection Kit, Dako, Glostrup, Denmark), and rinsing in PBS (3×5 min). After staining, the sections were counterstained with hematoxylin and dehydrated with ethanol and xylene before mounting on coverslips with Permount. To quantify the immunostaining, the optical density for CHOP and caspase-12 were calculated in the CA1 area of the hippocampal for all groups using Image-J software, after defining a threshold for background correction.

**Statistical analysis**

All statistical analysis was done with SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA). Group differences in the escape latency in the Morris water maze task were analyzed using one-way ANOVA with repeated measures. Data for the protein expression level, and the optical density of CHOP and Caspase-12-positive neurons, and the number of TUNEL positive neurons in hippocampus in different groups were analyzed by one-way ANOVA and Dunnett’s post-hoc test for the statistical analysis. Data are expressed as mean ± SD and a probability value less than 0.05 was considered statistically significant.

**Results**

*Effects of MTX on the memory and learning ability of the rats*

To evaluate the effect of MTX on the memory and learning ability of the rats, we subjected
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Figure 1. Rats treated with MTX performed more poorly in the Morris water maze test. A. Effects of MTX on escape latency to find the hidden platform. There were no significant differences between MTX-treated rats and controls in escape latency on the first day. On day 4, MTX group rats spent more time to arrive the platform than control group (P<0.05). B. The time the rats spent in the target quadrant. During re-training on the fifth day post treatment, MTX rats spent significantly less time searching in the target quadrant compared with the control animals (P<0.05). Data are presented as mean ± SD. n=10, *P<0.05, vs control group.

Effects of MTX on cellular morphology of hippocampal neurons in CA1 area

As compared with the control group, the number of neurons with nuclear pyknosis in MTX groups of the rats increased significantly in hippocampus CA1 area. Moreover, as compared to the MTX_60 group, the neuronal degeneration in MTX_100 and MTX_250 group was more severe. The neurons in control group displayed regular appearance with large and round nuclei but densely stained, and distorted cells with pyknotic appearance were observed in MTX_60, MTX_100 and MTX_250 group. A. control group; B. MTX_60 group; C. MTX_100 group; D. MTX_250 group.

Effects of MTX on the apoptosis of hippocampal neurons

We detected the apoptosis rate of hippocampus neurons in CA1 area with TUNEL stained. TUNEL positive cells were few in the control groups, and there were some TUNEL positive cells in the MTX_60 group (Figure 3A). However, the rats to MWM testing. As shown in Figure 1A, following four days of training, the latency to reach the platform in both of the groups significantly reduced during the learning phase, supported by a significant effect of training day (P<0.01). There were no significant differences between MTX-treated and controls rats in escape latency on the first day. On the fourth day, MTX group rats spent more time to arrive the platform than control group (P<0.05).

To observe memory retrieval, we analyzed the time that the rats spent in the quadrant fixed platform during the probe trial. As shown in Figure 1B, during re-training on the fifth day post treatment, rats treated with MTX appeared to perform worse than control rats, and MTX rats spent less time searching (27.3±3.98) the target quadrant as compared with the control animals (33.3±6.3) (P<0.05).

Effects of MTX on cellular morphology of hippocampal neurons in CA1 area

As compared with the control group, the number of neurons with nuclear pyknosis in MTX groups of the rats increased significantly in hippocampus CA1 area. Moreover, as compared to the MTX_60 group, the neuronal degeneration in MTX_100 and MTX_250 group was more severe. The neurons in control group displayed regular appearance with large and round nuclei but densely stained, and distorted cells with pyknotic appearance were observed in MTX_60, MTX_100 and MTX_250 groups (Figure 2).

Effects of MTX on the apoptosis of hippocampal neurons

We detected the apoptosis rate of hippocampus neurons in CA1 area with TUNEL stained. TUNEL positive cells were few in the control groups, and there were some TUNEL positive cells in the MTX_60 group (Figure 3A). However,
more TUNEL positive cells could be observed in the MTX\textsubscript{100} and MTX\textsubscript{250} group. The percentage of apoptotic hippocampal neurons in the control, MTX\textsubscript{60}, MTX\textsubscript{100} and MTX\textsubscript{250} groups were 5.47±2.71\%, 16.71±6.34\%, 36.85±3.85\% and 48.90±7.28\%, respectively. The number of TUNEL-positive cells in MTX\textsubscript{250} group increased nearly 9-fold more than the control group (Figure 3B). These findings indicate that MTX induced hippocampal neuronal death, as the dose of MTX increased, the apoptosis rate of hippocampus neurons increased ($P<0.01$).

**Effects of MTX on the expression of CHOP and caspase-12 in hippocampal tissues**

Western blot analysis showed that the expression levels of CHOP and caspase-12 in MTX\textsubscript{60}, MTX\textsubscript{100} and MTX\textsubscript{250} groups significantly increased as compared to the control group ($P<0.05$) (Figure 4A, 4C). Among the MTX\textsubscript{60}, MTX\textsubscript{100} and MTX\textsubscript{250} groups, as the dose of MTX increased, the expression levels of CHOP and caspase-12 increased ($P<0.01$ and $P<0.01$). However, there were no significant differences between MTX\textsubscript{100} and MTX\textsubscript{250} in the expression levels of CHOP and caspase-12 ($P>0.05$) (Figure 4B, 4D).

**Immunofluorescence staining of caspase-12 and CHOP in hippocampal tissues**

Immunohistochemistry indicated that CHOP and caspase12 were expressed predominantly in the nuclei and cytoplasm, respectively. As compared to the control group, the expression levels of CHOP and caspase-12 in the hippocampus of the MTX group were also significantly increased. While, the expression levels of CHOP and caspase-12 increased vividly in the MTX\textsubscript{100} and MTX\textsubscript{250} groups as compared to the other two groups (Figure 5A). Quantitative analysis indicated that the expression levels of CHOP and caspase-12 in the hippocampus were increased in the MTX\textsubscript{100} and MTX\textsubscript{250} groups as compared to the control rats (13.83±3.66 and 12.83±3.06 respectively) ($P<0.05$). Meanwhile, the expression levels of CHOP and caspase-12 increased to 191.88±17.78 and 219.88±19.97 in MTX\textsubscript{100} group respectively, while they increased to 203.537±18.99 and
230.37±19.51 in the MTX250 group as compared to the MTX group respectively. However, there were also no significant differences between MTX100 and MTX250 in the optical density for CHOP and caspase-12 (Figure 5B, 5C).

Discussion

According to some recent clinical researches, most of the ALL patients may present with different degrees of impairments in attention, verbal and visual memories after chemotherapy [14]. Besides, several experimental studies have demonstrated that MTX may contribute to memory impairment by inducing apoptosis and inhibiting proliferation of hippocampal cell in the rat [15-17]. Furthermore, ERS-related neuronal apoptosis plays an important role in various kinds of neurodegenerative diseases, however, there is few finding about the role of ERS played in MTX-induced neurotoxicity and cognitive dysfunction. Therefore, we hypothesized that ERS either mediated the apoptosis of hippocampal neurons or induced memory impairment after the administration of high-dose MTX in adult rats.

In order to assess the effects of MTX on the memory and learning ability of rats, a widely used Morris water maze test was implemented in this study. In the Morris water maze test, we observed that MTX-treated rats took longer to find the escape platform location and shorter time to swim in the escape platform site compared to controls (Figure 1A, 1B). These results are similar to those of previous studies in which animals treated with MTX showed a defect in cognitive performance [6]. Therefore, we could conclude that MTX do induce impairment in spatial learning and recognition memory.

In several previous studies cognitive impairments due to chemotherapy treatment have been found to be associated with hippocampal dysfunction. Hippocampus belongs to the limbic

![Figure 4. MTX increased hippocampal expression of CHOP and caspase-12. Western blot analysis of the protein expressions of CHOP and caspase-12 for the control, MTX60, MTX100, and MTX250 groups are shown. GAPDH was used as the loading control and for band density normalization (A, B). Graphs indicate the relative band intensities compared with GAPDH (C, D). *P<0.05, vs the control group. #P<0.05, vs the MTX group. Data are presented as means ± SDs, n=6.](image-url)
bic system and associated with affective, cognitive and behavioral functions. The major result of the present study is that MTX treatment significantly increased the number of degenerated neuronal and the percentage of TUNEL positive neurons in hippocampus CA1 area (Figures 2 and 3). This is consistent with previous studies that discovered the number of neurons and neuroglial cells decreased in the CA3, CA4 of the hippocampus after intrathecal injection of MTX in rats [18]. Further, the present results also show that the hippocampal cell apoptosis increased as the dose of MTX increased. These findings indicated that methotrexate-related cognitive deficits may be associated with the apoptosis of hippocampus neurons.

Increasing evidences demonstrated that caspase-12 and CHOP are both important mediators of ERS apoptosis pathway [19, 20]. MTX could activate the unfolded protein response in the promyelocytic leukemia cell line HL60 cells by regulating the expression of ER-resident proteins in a time dependent manner [21]. MTX may induce apoptosis through production of reactive oxygen species (ROS) leading to oxidative stress [22], moreover, as ROS accumulation, endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) were activat-
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Ed, accompanied by an up-regulation of CHOP expression, cleavage of poly (ADP-ribose) polymerase (PARP) and increase of cell apoptosis [23-26]. In the present study, we found that MTX exposure could up-regulate the protein expression of CHOP and caspase-12 in the hippocampus of adult rats, and there were no significant differences between MTX100 and MTX250 groups in the protein expression level (Figures 4 and 5). These results revealed that MTX-induced neuron apoptosis may be related to the ERS and, there is no obvious different in the protein expression of CHOP and caspase-12 between MTX100 and MTX250 group. However the protein expression of CHOP and caspase-12 was not in conformity with TUNEL statistical results. The percentage of TUNEL positive neurons of MTX250 group was more than the other three groups. It suggested that other apoptosis mechanism may involve in high-dose MTX-induced hippocampus apoptosis. Therefore, we need further studies for exploring it.

In summary, the present study demonstrated that MTX could induce hippocampal neuronal apoptosis and lead to cognitive impairment in adult rats through ERS pathway.

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Disclosure of conflict of interest

None.

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