Original Article

Protective Effect of Low Dose of Methamphetamine on The Amount of Extracellular Glutamine in Primary Fetal Human Astrocytes Induced by Amyloid Beta

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Abstract

Objective: Change in astrocytes is one of the first pathological symptoms of Alzheimer's disease (AD). Understanding the signaling pathways in astrocytes can be a great help in treating of AD. This study aimed to investigate signaling pathway relations between low dose of methamphetamine (METH), the apoptosis, cell cycle, and glutamine (Gln) pathways in the activated astrocyte.

Materials and Methods: In this experimental study, the activated astrocyte cells were exposed to a low dose of METH (12.5 μM) which was determined by Thiazolyl blue tetrazolium bromide (MTT) method. The groups were: group 1 cells with Aβ, group 2 cells with METH, group 3 cells with METH after 24 hours of adding Aβ (Aβ+METH, treated group), group 4 cells with Aβ after 24 hours of adding METH (METH+Aβ, prevention group), and group 5 as the control. The Gln was assayed by high-performance liquid chromatography (HPLC), and also the apoptosis, and cell cycle and *BAX, BCL-X* expression was evaluated.

Results: The amount of Gln was increased, and the value of late and early apoptosis was reduced in the treatment groups, and necrosis is decreased in the prevention group (group 4 compared to group 1). Moreover, it was revealed through cell cycle analysis that G2 in group 4 was reduced compared to group 1 and the expression of *BAX, BAX/BCL-X*, and *BCL-X* in group 3 and group 4, was decreased and increased, respectively compared to group 1.

Conclusion: These findings suggest that perhaps a non-toxic dosage of METH (low dose) can reduce the amount of apoptosis and *BAX* expression and increase the expression of *BCL-X*. Furthermore, the cells are arrested in the G2 phase and can raise the amount of extracellular glutamine, which has a protective role in neuron cells. These findings may provide a new perspective to design a new drug with less toxic results.

Keywords: Alzheimer's Disease, Astrocytes, Cell Cycle, Glutamine, Methamphetamine

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Introduction

The presence of astrocytes is essential to keep up homeostasis in the brain. They are usually involved to maintain the nervous system and improve neurodegenerative diseases such as Alzheimer's disease (AD) (1). The important roles of astrocytes in the central nervous system (CNS) are: as follows establish homeostasis through regulation pH, recycling oxidized ascorbic acid, reserve lactate to neurons and ATP production, homeostasis of Ca^{2+}, production of glutathione, and osmolality adjustment (2). Astrocytes are actively involved in maintaining and protecting the CNS microenvironment in normal and pathophysiological position and participating in oxidative stress (3). Astrocytes produce glutamine (Gln) through glutamine synthetase (GS), Gln gain has a protective role in AD in response to injury or toxic substance (4). Astrocytes were activated and leads to hypertrophy and ramification process. Reactive astrocytes participated in inflammatory response and pathogenesis of AD (5).

Activation hypertrophy and ramification of astrocytes occurred after neurodegenerative diseases or traumatic brain injury. The GS is a specific astrocyte enzyme which could be converting glutamate (Glu) and ammonia to Gln. This enzyme was reduced in AD (6). In a senescence astrocyte, loss of synaptic plasticity, blood-brain barrier (BBB) dysfunction and Glu excitotoxicity were caused (7). Astrocytes can balance neuronal function by uptake of Glu and γ-aminobutyrate (GABA) and stimulates released from synapses. Glu is one of the neurotransmitter take part in memory and learning. Glutamate/aspartate transporter (GLAST) and Glutamate transporter-1 (GLT-1) absorbed Glu into astrocyte and GS converted Glu to Gln (8).

Oxidative stress acutely affects the activity of GS (9). Senile astrocyte capacity was reduced in neurodegenerative diseases (10). Accumulation of Gln in astrocytes leads to mitochondrial dysfunction and cell swelling. Ammonia detoxification due to the amidation of Glu to Gln (11). Neurons by Glu/Gln cycle absorb Gln and clear Glu from the synaptic cleft by astrocytes and convert it to glutamine. The stress response proteins

were reduced in a low concentration of Gln and cells will become hypersensitive to H_2O_2 and DNA damage (12).

Aβ deposition, chronic inflammation, hypoxia, ischemia and oxidative stress can directly reduce GS activity. Gln deficiency blocks mitochondrial energy production, DNA damage response, apoptosis, and autophagy (13). Aβ induces apoptosis in the cell cultures of neuron and the brain of transgenic mice; on the other hand, Aβ reduced expression of anti-apoptotic *BCL-X* significantly (14). Astrocytes are significant mediators in the neurotoxicity of AD and participated in neuronal death regulation induced by Aβ (15, 16).

METH is an incentive and major addiction in high repetitive doses in the world. It was used to treat attention deficit hyperactive disorder (ADHD), obesity, and narcolepsy (17). Recent evidence indicates that some herbal ingredients such as crocin, picrocrocin and safranal are neuroprotective (18). Moreover, low doses of METH (IV infusion with 0.5 mg/kg/h for 24 hours) can produce neuroprotection (19). Thus, it appears that METH under certain circumstances and correct dosage can produce a neuroprotective effect (4, 19, 20). It presents an interesting paradox of neuroprotection and neurotoxicity (21) effect of METH that needs further investigation *in vivo*. The appropriate (low) dose of METH was determined by the MTT method. Half-maximal inhibitory concentration (IC_{50}) was 25 μM, to evaluate the therapeutic effect of METH, we used a lower dose than IC_{50} (12.5 μM). Gln is an apoptosis suppressor and may be a protective effect on cells from stress (3). Also, Gln enriched-diet significantly enhances the expression of *BCL-X* (22). We intent to use astrocytes because the activation of astrocytes is one of the first findings in the brains of people who abused METH (23). Due to the findings of the anti-apoptotic effect of Gln in the past, we investigated a little dose of METH on astrocytes induction oxidative stress by exposure to Aβ (24) to understand the relationship between the amount of Gln in the supernatant of treated astrocyte, apoptosis, and expression of *BAX*, *BCL-X* genes. The novelty of the above research investigates the relationship between the effective dose of METH and the amount of Gln and the relationship of these pathways.

Materials and Methods

All animals used in the study were handled in accordance with the guidelines approved by the Ethics Committee of our University with approval ID: IR.IAU.DAMGHAN.REC.1398.005.

Preparation of Aβ1–42 peptide

In this experimental study, Aβ1-42 peptides are dissolved in some hexafluoro-2-propanol (HFIP, Sigma-920-66-1) to reach a final concentration of 1 mM (monomer). To evaporate the HFIP using a Speed Vac, the samples were stored at -20˚C until to use. For fibril formation, the large aggregates Aβ of the tube were directly dissolved in dH_2O and incubated at 37˚C for 72 hours.

Astrocytes culture and treatment

Primary fetal human astrocytes were isolated from the hypothalamus and cerebral cortex, which were previously isolated from hypothalamus and cerebral cortex of two human fetuses on gestational weeks 9-12 (gift from Bon Yakhteh Laboratory in Tehran) were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (50 mg/mL the cells were incubated at 37˚C in 5% CO_2, 85-95% humidity. 200000-250000 cells were cultured in each well (25). According to the IC_{50}, after 24 hours, METH (donated by Tehran University) and Aβ were added to the well. METH was added to DMEM, containing 10% FBS, to reach the final concentration of 12.5 μM. METH remained in the vicinity of the cell for 24 hours. For treatment with Aβ, 10 μM of Aβ was kept at 37˚C for 72 hours (fibril formation) and then added to DMEM plus F12 without FBS (26). Cells were exposed to amyloid for 24 hours. All experiments have been performed according to the following procedures: group 1 cells with Aβ, group 2 cells with METH, group 3 cells with METH after 24 hours of adding Aβ (Aβ+METH, treated group), group 4 cells with Aβ after 24 hours of adding METH (METH+Aβ, prevention group) and group 5 as control.

Cell viability by MTT assay

Astrocytes were seeded in a 96-well plate (10000, 15000, and 20000 cells per well) with 5% FBS in DMEM, and exposed to various concentrations (0.8, 1.6, 3.12, 6.25, 12.5, 25, 50, and 100 μM) of METH for 24 hours, 48 hours, and 72 hours. Then, MTT [Sigma-Aldrich, USA, 5 mg/mL in phosphate-buffered saline (PBS, Jenabioscience, Germany)] was added. Dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) was used to solubilize the crystals and the absorbance was measured at 570 nm (27).

Chromatographic system

The chromatographic conditions were used Waters 2795, fluorescence detector Waters474 and C18 column (250×4.6 mm, 10 μm) Empower software system and retention time (RT) condition. 0.4% tetrahydrofuran with 30 mmol/L potassium dihydrogen phosphate with pH=7.0 (adjust with 4 mol/L KOH) used as mobile phase A, and mobile phase B was acetonitrile 50%. All mobile phases were filtered by 0.22 μm filter and were degassed. The 340 nm and 455 nm wavelength respectively used as excitation and an emission wavelength. The volume was 10 μl. The gradient conditions were based on the previous study (28).

Standard solution

The standard solution was prepared similar to the sample in water, stored at -70˚C. The linearity range of the proposed method was 5 to 1000 μmol/L. The concentration of amino acid calculated by divided the area of sample to the area of internal standard. The correlation coefficients (r) were >0.99.

Sample preparation

For prepared 200 µl sample, 180 µL of high-performance liquid chromatography (HPLC) grade water was added to 20 µL sample. Then, 200 µl of methanol HPLC grade was added to precipitate protein. The samples were centrifuged for 5 minutes at 10,000 × g in RT. The supernatants were collect and mixed with derivatization reagent and incubated for 30 minutes before injection.

Apoptosis and necrosis assay

Annexin-V-FITC/PI immunostep kit (ANXVF-200T) was used to separate necrotic cells from apoptotic cells. Briefly, pre-treated astrocyte cells were harvested by trypsin and washed twice with PBS 0.01 M. After that, the cells centrifuge for 5 minutes at 2000 rpm, the cellular deposition was re-suspended in 500 µl of binding buffer; the density of cells must be 1×10^6 cell/ml. Following that, 5 µl Annexin, V-FITC, and 5 µl propidium iodide (PI) were added, respectively. Cells were incubated in the dark at 25˚C for 15 minutes and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Analysis of cell cycle

The pre-treated astrocyte cells were harvested by trypsin and washed twice with PBS after the cells centrifuge for 5 minutes at 2000 rpm, at 4˚C. The cells were resuspended in cold PBS (DNase- RNase-free Sigma) and stained with PI containing 1% Triton X-100 (v/v) (Sigma, USA). The solution was incubated at 20˚C for 30 minutes (preserved from light) and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) (29).

RNA extraction

According to the manufacturer's recommendation, total RNA of astrocyte culture was extracted by Roche RNA extraction kit (Roche 11828665001). By optical density (OD) at 260 nm, concentration of RNA samples was determined and by detecting 18S and 28S bands on agarose gel electrophoresis, the quality of RNA was confirmed. The RNA samples were incubated with DNase at room temperature for 15 minutes to remove residual DNA contamination.

cDNA synthesis

According to the manufacturer's protocol Thermo (K1621), cDNA was generated with oligo (dT) primers from the total RNA. Oligonucleotide primers *GAPDH* was used for housekeeping genes, and primer 3 was applied to design all primers. The primer of:

GAPDH-

F: 5′-GACCACTTTGTCAAGCTCATTTCC-3′
R: 5′-GTGAGGGTCTCTCTCTTCCTCTTG-3′ (168 bp)

BAX-

F: 5′-TGGAGCTGCAGAGGATGATTG-3′
R: 5′-GAAGTTGCCGTCAGAAAACATG-3′ (98 bp)

BCL-X-

F: 5′-CTGAATCGGAGATGGAGACC-3′
R: 5′-TGGGATGTCAGGTCACTGAA-3′ (211 bp) have been used.

Quantitative real-time polymerase chain reaction analysis

The *BCL-X* and *BAX* genes related to apoptosis were evaluated using the housekeeping gene (*GAPDH*). The primers were previously checked by conventional reverse transcription polymerase chain reaction (RT-PCR) and agarose gel (1.5%) electrophoresis. Quantitative PCR (Q-PCR) was performed using the Amplicon PCR Master Mix (A314406) and Qiagen Rotor-Gene Q system. All experiments were performed in triplicates. After an initial denaturation step of 3 minutes at 94˚C, 35 cycles of amplification were carried out. Each cycle included a denaturation step, 30 seconds at 94˚C; an annealing step, 30 seconds at 60˚C; and an elongation step, 45 seconds at 72˚C. The final elongation temperature was 72˚C for 5 minutes. The fold-change in gene expression was calculated using the melt curve method and was normalized to *GAPDH* then the relative gene expression levels were calculated with reference to the control (30).

Statistical analysis

SPSS v16 (Chicago, USA) and GraphPad Prism8 (San Diego, California, USA) were used for statistical analysis. Each of the treatment groups was compared with a group using sample t test in real-time PCR. $P<0.05$ was set as the level of significance. All error bars in the figures are based on the results of the mean ± standard deviation (SD). Each experiment was performed in triplicate.

Results

Viability of astrocyte in the presence of a different concentration of METH

MTT assay was used to determine the number of viable cells in exposure to METH. The cells were incubated with different concentrations of METH for 24, 48, and 72 hours. It was revealed the cell viability reduction (~ 10%) in the astrocytes within the limited concentrations of METH (0.8, 1.6, 3.1, 6.2, and 12.5 µM) for 24, 48, and 72 hours. Therefore, 12.5 µM concentration of METH was employed for further evaluation (Fig.1).

Glutamine analysis by HPLC

At the end of the treatment, the concentration of Gln in the supernatant of samples was assayed by HPLC. The concentration of Gln in group-3 (Aβ+METH) and group-4 (METH+Aβ) was amplificated in comparison with group-1 (Aβ) as shown in (Fig.2).

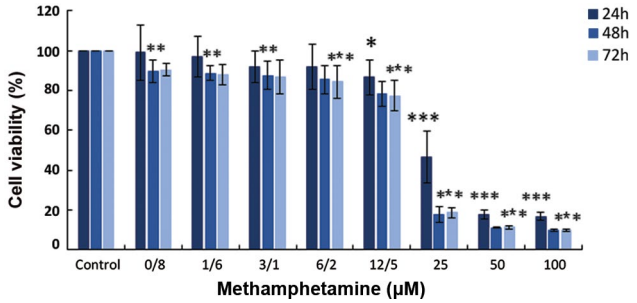

Fig.1: Effect of methamphetamine (METH) on astrocytes viability. The cells are treated with different concentrations (0.8-100 µM) of METH for 24 hours, 48 hours, 72 hours (n=3). Data values are expressed as % of control values. Significant change (*0.01<P<0.05, **0.01<P<0.001 and ***P<0.001) in comparison with control group). Concentration 12.5 µM of METH (*0.01<P<0.05), 25, 50, and 100 µM of METH (***P<0.001) in 24 hours. Concentration of (0.8, 1.6, 3.1 µM of METH (**0.01<P<0.001) and 6.2, 12.5, 25, 50 and 100 µM (***P<0.001) in 48 and 72 hours. The results show that 12.5 µM METH for 24 hours, 48 hours, and 72 hours has a 10% toxicity effect on astrocytes. The data presented as mean ± standard deviation (SD).

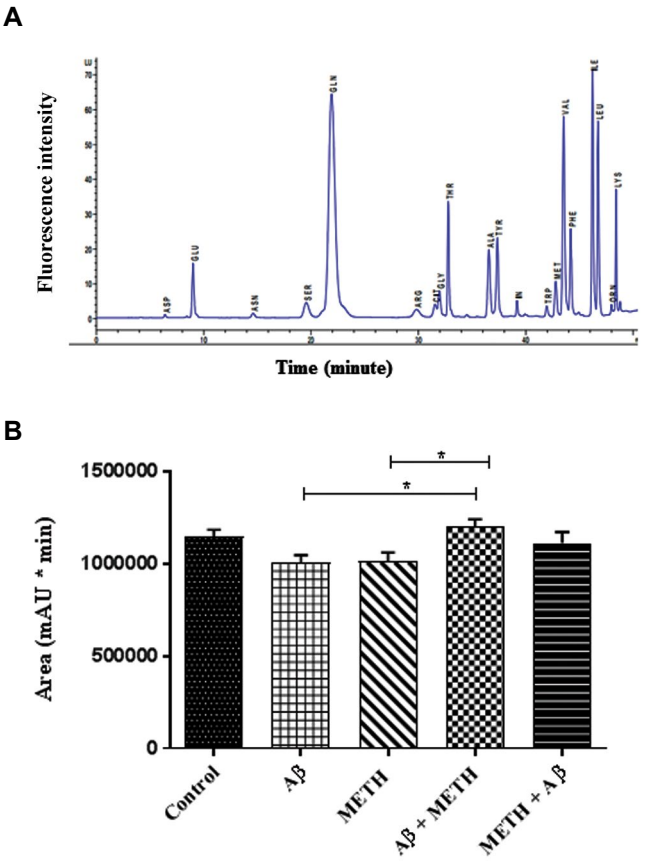

Fig.2: Glutamine analysis by HPLC. **A.** Glutamine analysis by HPLC. Group 1 (Aβ), group 2 (METH), group 3 (Aβ+METH), group 4 (METH+Aβ) and group 5 (control). The amount of glutamine in the supernatant of groups (Aβ+METH) and (METH+Aβ) was increased in comparison with a group (Aβ). **B.** The area of glutamine in astrocyte treated with a low dose of METH, The comparison between Aβ and (Aβ+METH) as well as METH and (Aβ+METH) was significant (*0.01<P<0.05). HPLC; High-performance liquid chromatography and METH; Methamphetamine.

Apoptosis and necrosis analysis

The flow cytometry method was adopted to determine the number of live cells, late apoptosis, early apoptosis, and necrosis. The percentage of early apoptosis decreased in Aβ+METH (2.33%) and METH+Aβ (2.93%), compared to the Aβ group-1 (Aβ). Also, 91.5 and 90.3% of live cells arise in METH+Aβ and Aβ+METH groups compared to the Aβ group. The percentage of live cells rose in all treatments compared to Aβ. The amount of (early and late) apoptosis reduced in all groups in comparison to the Aβ group (Fig.3).

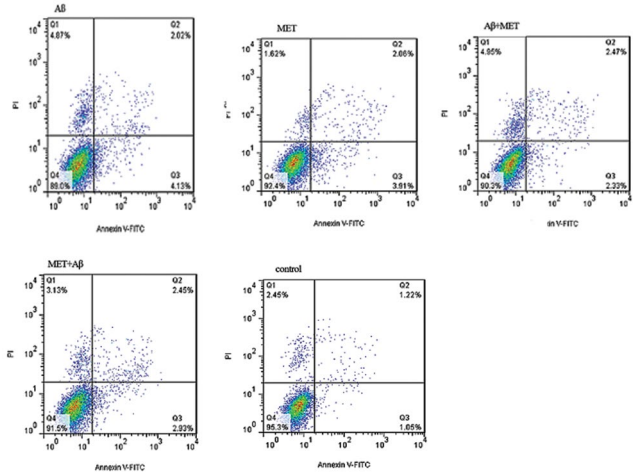

Fig.3: Evaluation of apoptosis in astrocytes in the presence of low dose of methamphetamine by flow cytometry. The control group shows the untreated astrocyte cells after 72 hours, (Aβ) group treated with 10 µM Aβ for 24 hours, (METH) group after astrocyte was treated with 12.5 µM METH for 24 hours, (Aβ+METH) group astrocyte which was treated for 24 hours with Aβ and then 24 hours with METH, (METH+Aβ) group that astrocyte was treated with METH for 24 hours and after that treated with Aβ for 24 hours, and the data was analyzed in both treated and untreated group. Q1; PI=Positive, Annexin V FITC negative (necrosis), Q2; PI=Positive, Annexin V FITC positive (late apoptosis), Q3; PI=Negative, Annexin V FITC positive (early apoptosis), Q4; PI=Negative, Annexin V FITC negative (live cell) (all tests were repeated twice), and METH; Methamphetamine.

Cell cycle analysis

Comparison of the results in different treatment groups showed that in group-1 (Aβ), the cells enter the S phase and arrest in G2 in comparison with the control group. In Aβ+METH group, astrocyte cells enter the S and G2 phases, G1 decreases, and the cells arrest in G2, compared to the Aβ group phase. In METH+Aβ group, the cells arrest in G2 and enter S compared to the Aβ group (Table 1, Fig.4).

Table 1: Cell cycle arrest in all groups, group 1 (Aβ), group 2 (METH), group 3 (Aβ+METH), group 4 METH+Aβ) and group-5 (control)

Groups	G1 (%)	S (%)	G2 (%)
Aβ	64.45	19.35	14.35
METH	63.92	21.71	11.8
Aβ+METH	44.07	29.76	22.64
METH+Aβ	66.44	13.43	13.05
Control	70.54	15.57	8.02

In group 3 and 4 the % of G2 w ere increased in comparison with group 5 control group. G1; Intermediate phase occupying the time between the end of cell division in mitosis, G2; Checkpoint prevents cells from entering mitosis, S; Stands for DNA synthesis, and METH; Methamphetamine.

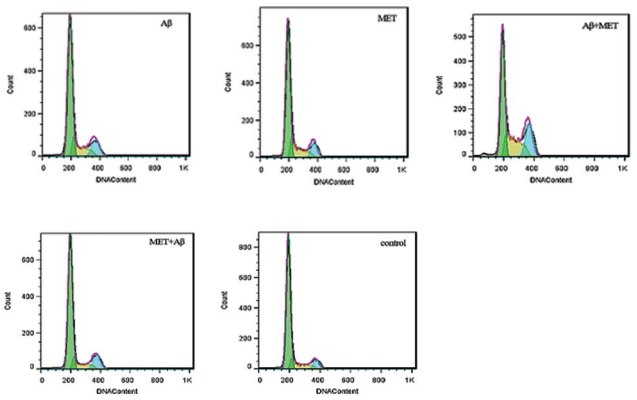

Fig.4: The effect of different treated METH on the cell cycle arrest group 1 (Aβ), group 2 (METH), group 3 (Aβ+METH), group 4 (METH+Aβ), and group-5 (control). G2 increases and G1 decreases in group-1. In group 4, G2 increases and G1 decreases, the amount of G2 decreases in group 3 and G1 increases, all groups compared to group-1 (all tests were repeated twice). METH; Methamphetamine.

Gene expressions *BAX* and *BCL-X*

Expressions of these genes were measured by RT-PCR in experimental groups. *BAX* expressions in group 3 (Aβ+METH) decreased significantly (P=0.035) despite an increase in *BCL-X* which was not significant compared to group 1 (Aβ). *BAX* in group 4 (METH+Aβ) decreased considerably (P=0.001) and *BCL-X* expression increased (P=0.048) compared to group 1 (Aβ). The ratio of *BAX/BCL-X* in group 3 (Aβ+METH) reduced about 0.432 fold (P=0.023) compared to group 1 (Aβ), while the ratio of *BAX/BCL-X* in group 4 (METH+Aβ) decreased about 1.17 fold (P=0.047) compared to the same amount in group 1 (Aβ) (Fig.5A-F).

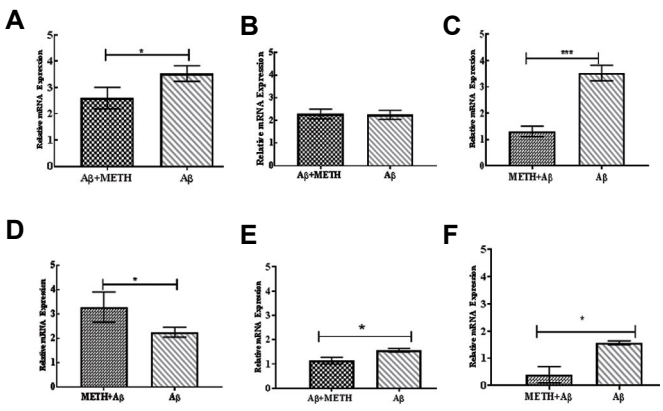

Fig.5: Real-time PCR analysis for *BAX* and *BCL-X* genes. The values put onto each graph represent the relative fold change calculated by calibrating the ΔΔCt data *BAX* and *BCL-X* gene expressions in several experiments. **A.** *BAX* in group 3 (Aβ+METH) and **B.** Group 4 (METH+Aβ) are decreased (P<0.001 and P<0.05, respectively). **C.** *BCL-X* in group 3 (Aβ+METH), **D.** Group 4 (METH+Aβ) are increased (P<0.001) and **E, F.** *BAX/BCL-X* in group 3, 4 are decreased in comparison with group 1 Aβ. Error bars indicate SEM. The significant level was define as *P≤0.05 and ***P<0.001. Group 1 (Aβ), group 2 (METH), group 3 (Aβ+METH), group 4 (METH+Aβ).

Discussion

We examined the hypothesis that astrocytes respond to a low dose of METH exposure by raising the amount of extracellular glutamine, as an indicator of neuroprotection, increase in extracellular Gln neurons. Our findings show that the amount of Gln increased in treatment with a low dose of METH in treated (Aβ+METH) and prevention (METH+Aβ) groups in comparison to the AD model or group 1 (Aβ). The cells in low concentrations of Gln are sensitive to stress oxidative and DNA damage. The value of proteins responding to stress was reduced as a result, and cells are more sensitive to the neurotoxic effects like Aβ. As well as Gln supplements have a neuroprotective effect on AD (12).

The Gln/Glu levels have been reduced in the AD brain, which confirmed our observations. One of the early signs of AD is a decrease in Gln levels (31). The present findings suggest that Gln in the AD model is lower than in the groups treated with METH. In previous studies, the neuroprotective effect of a low dose of METH was dependent on a PI3K/AKT pathway. Notably, the activation of the pathway PI3K/AKT suppresses apoptotic factors (32).

In our study, the effect of low concentration of METH on apoptosis of astrocyte cells in two treatment patterns was evaluated. Our results show that in group 3 and group 4, live cells were amplificated compared to activated astrocytes. Early and late apoptosis in all treatments were rebates, but the rate of necrosis was reduced only in the pretreatment position. Earlier research shows that mild stress can prevent the occurrence of larger ones. In this respect, researchers used METH with concentration 0-3 mM to provide mild stress and 6-hydroxydopamine (6-OHDA) as a potential source of toxic stress. The prior research has indicated that previous exposure to nontoxic concentrations of METH protected these cells against 6-OHDA toxicity, but higher concentrations of METH intensify it (33).

In our study, we explained two models for the treatment. In one group, there was astrocyte exposure with Aβ, and after 24 hours effective dose of METH (treatment group) was added. In another group, the cells were exposed to a little dose of METH, and then after 24 hours, Aβ was added (prevention group). In both models, we checked the therapeutic effects of METH on apoptosis and cell cycle in cell signaling pathways The apoptosis results in our study also show that the number of live cells was increased in the inhibition form (group 4) compared with the treatment form (group 3). It may be the effect of mild stress-induced by METH which can protect against the larger ones. The other effects of METH exposure were the up-regulate of the *BCL-2*. Exposure to low concentrations of METH causes many dopamine changes, alike decrease in their vulnerability to further oxidative stress (33).

Our findings show that the expression of *BCL-X* was enhanced in the prevention groups more than it did in the treatment form (group 4). Bile duct ligation (BDL)

in rodents can cause cognition deficits and treatment with Curcumin has a preventive and therapeutic role in memory impairment. Curcumin increased expression of the *BCL-X* and decreased the *BAX* gene expression level (34).

We observed that the expression level of *BAX* in both groups decreased through the representative data, but in the METH+Aβ group, the reduction was more than the Aβ+METH group. This finding may show that a slight increase in METH in the prevention mode may reduce *BAX* more efficiently than that in the treatment mode. As soon as neurons are born, they lose the capacity of division and differentiation. In stress conditions, like oxidative stress and DNA damage, and after neuronal differentiation, cell cycle modulators' expression increased (35). The cell cycle consists of four main phases: G1, S, G2, and M. Neurons remain in a G0 phase. G0 is a nondividing and nonreplicating phase, where cell division is initiated but not completed; it finally enters apoptosis. Before the neurons die, cell cycle abnormalities in AD may be arrested at the G2/M (36).

On the other hand, we analyzed the cell cycle in activated astrocytes treated with a low dose of METH. The results show that in prevention mode, G2 was reduced compared to AD, but increased in the treatment mode importantly, susceptible neurons before die may be arrested at the G2/M. Thus, the rate-limiting step before apoptosis of neurons might activate CDK1 at G2. Phosphorylation of tau during G2 is a direct link between the cell cycle and the cell death (37). Moreover, tau can be phosphorylated by CDK1 (38). Abnormally increased levels of tau phosphorylation cannot modulate G2 and prepare for mitosis. Initiators of the cell cycle can play major roles in treating AD and be regarded as a therapeutic target.

The previous study suggested insulin signaling impairment and glucose metabolism reduction in AD patients (39). In previous studies, low concentrations of METH (20 mM) on increasing glucose receptors have been investigated. METH exposure showed a dual effect on the uptake the glucose in astrocyte, in the concentration of 20 μM increases the uptake and in 200 μM inhibit the uptake of glucose (40). Perhaps the desired effect of low-dose of METH to improve the cognitive effects of AD was related to the increased glucose receptors, which requires further research. Thus, our findings provide a new perspective for understanding apoptosis's molecular mechanism, cell cycle, and Gln in reactive astrocytes.

To further confirm the protective effect of methamphetamine, more clinical trials are needed. Because of the lack of time and cost, we could not do it. It would have been better that method validation and system suitability was done for the HPLC method, that only assay standardization was performed.

Conclusion

Our results showed that since astrocytes are the most important supporter cells in the CNS, despite the effects of high-dose METH, low-dose of METH can reduce apoptosis rate induced by Aβ. It also affects the cell cycle and the cell arrested in the G2 phase. Therefore, an effective dosage of METH can increase the amount of extracellular glutamine, which has a protective role in neuron cells, as well as the amount of *BAX* and *BCL-X*, which respectively decreased and increased the expression of these genes. Although METH has the effects of addiction; on the other hand, due to the effects of the low dose of METH for the prevention and treatment of AD, a drug can be designed that has a protective effect but has no side effects as METH.

Acknowledgments

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Authors' Contributions

B.S.; Performed all *in vitro* experiments, analyzed the data, and wrote the manuscript. M.D.S., G.H.R.; Contributed to concept and design, financial support, analyzed the data, and wrote the manuscript. N.M.; Analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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