Alteration of gene expression in reactive astrocytes induced by Aβ1-42 using low dose of methamphetamine

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Abstract
Background Alzheimer’s disease (AD) is a degenerative brain disorder. Due to the relationship between the functional loss of astrocytes and AD, the present study aims to evaluate the effects of the low dose of methamphetamine (METH) on primary fetal human astrocytes under a stress paradigm as a possible model for AD.

Methods and results The groups in this study included Aβ (Group 1), METH (Group 2), Aβ + METH (METH after adding Aβ for 24 h) (Group 3 as treated group), METH + Aβ (Aβ after adding METH for 24 h) (Group 4 as prevention group), and control group. Then, the gene expression of Bax, Bcl-X, PKCα, GSK3β, and Cdk5 was evaluated. In addition, phosphorylated tau, p-GSK3β, GSK3β, and GSK3α proteins were assessed by western blotting. Further, cell cycle arrest and apoptosis were checked by flow cytometry and Hoechst staining. Based on the results, the expression of GSK3β, Cdk5, and PKCα genes decreased in the prevention group, while GSK3β and Cdk5 were amplified in the treatment group. Furthermore, the level of GSK3α and GSK3β proteins in the treatment group increased, while it decreased in the prevention group. Additionally, a decrease occurred in the percentage of necrosis and early apoptosis in the treatment and prevention groups. The results of the cell cycle indicated that G1 increased, while G2 decreased in the prevention group.

Conclusion The pure form of METH can prevent from activating GSK-3β and CdK-5, as well as enhanced activity of PKCα to inhibit phosphorylated tau protein. Therefore, a low dose of METH may have a protective effect or reducing role in the pathway of tau production in reactive astrocytes.

Keywords Alzheimer’s disease · Apoptosis · Hyperphosphorylated tau · Methamphetamine

Introduction
Alzheimer’s disease (AD) is the most frequent neurodegenerative condition which causes dementia in elderly people all over the planet [1, 2]. People with AD have two major pathological symptoms including extracellular amyloid plaques and intracellular neurofibrillary tangles (tau protein) [3]. Amyloid beta (Aβ) performs a critical part in AD pathogenesis. Aβ may be found in astrocytes and microglia although it leaves the neurons [4]. Astrocytes are the most abundant glial cells, as well as the scavenging cells in the brain. In addition, they play a significant role in the pathogenesis of AD [5], the functions of which can influence neural survival. Further, they can support neurons in many different ways including energy production of the brain, ion, pH balance, synapse formation, remodeling, and oxidative stress regulation [6]. These glial cells participate in clearing Aβ in vitro and play a key role in the early stages of AD [7]. Reactive astrocyte is considered as one of the signs of many neurodegenerative diseases like Parkinson’s and AD. Reactive astrocytes are activated forms of astrocytes in response to toxic materials, as well as taking up amyloid-beta toxins. In addition, reactive astrocytes are identified in the vicinity of amyloid plaques and can be monitored by the increased expression of the intermediate filament protein, GFAP. Astrocytes are essential for an adequate brain function. In AD, astrocytes become reactive, which are associated with the deposit of AB [8]. Progress in the knowledge...
of molecular mechanisms of astrocytes may lead to the development of novel therapeutic strategies for neurodegenerative disorders. Aβ-induced synaptic dysfunction reduces tau phosphorylation by activating protein kinase B (AKT), which inhibits glycogen synthase kinase 3 (GSK3β) [9]. Tau is considered as a microtubule-associated protein (MAP), which is expressed in astrocytes and neuritis, which plays a role in regulating microtubule stability [10]. Tau phosphorylation is an early development in AD progression [11]. Aβ1-42 monomers and oligomers alter the phosphorylation of tau protein [9]. The protein kinase C (PKC), as one of the enzymes, is related to amyloid precursor protein (APP) [10, 12]. Regarding the neurons among AD patients, the first abnormality is related to the defect in PKC signal channels. Inhibiting PKC activity leads to the reduction of learning and memory capacity [13]. The activation of PKC inhibits the activity of GSK3 and hyperphosphorylation of tau in serine 9 [14]. GSK3β inhibition decreases the production of Aβ in the Alzheimer’s mouse model, as well as Aβ in the neuronal cell culture [15]. Reactive astrocytes encircling Aβ plaques take part in topological inflammatory responses and modulate calcium signaling [16]. Astrocyte activation is considered as one of the first findings in the brain of people who abused METH [17]. Based on recent evidence, 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 h) can produce neuroprotection [19]. A safe and secure dose of − 0. Methamphetamine (METH) probably has a protective effect on neurons and astrocyte cells [18, 19]. Thus, the present study aimed to evaluate the alteration and association between important enzymes such as PKCa, GSK3β, and Cdk5 in the reactive astrocyte induced by Aβ1-42 in the presence of a low dose of METH.

Materials and methods

All samples used in the study were collected 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

Aβ1-42 peptides were monomerized by dissolving in Hexafluoro-2-propanol (HFIP) (Sigma-920-66-1) for preparing the 1 mM concentration of Aβ. Then, 2.2 ml of HFIP was evaporated by using a Speed Vac, and the peptide was stored at − 20 °C until use. To fibril formation, large aggregates of Aβ1-42 were directly dissolved in dH2O and incubated at 37 °C for 72 h [20]. All other chemicals were of analytical reagent grade.

Astrocytes culture and treatment

Primary fetal human astrocytes were isolated from the hypothalamus and cerebral cortex, which were previously isolated from the 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) were purchased from Sigma Chemical Co. (Sigma-F2442, St. Louis, USA), and kanamycin (50 mg/ml) of the cells was incubated at 37 °C in 5% CO2, 85–95% humidity. Then, 200,000–250,000 cells were cultured in each well [21]. Based on the IC50, METH (donated by Tehran University) and Aβ were added to the well after 24 h. METH containing 10% FBS was added to DMEM to reach the final concentration of 12.5 μM. In addition, METH remained in the vicinity of the cell for 24 h. Regarding the treatment with Aβ, 10 μM of Aβ was kept at 37 °C for 72 h (fibril formation) and then added to DMEM plus F12 without FBS [22]. The cells were exposed to amyloid for 24 h. Further, all experiments were performed as follows. Cells with Aβ (Group 1), cells with METH (Group 2), cells with METH after 24 h of adding Aβ (Aβ + METH, treated group), cells with Aβ after 24 h of adding METH (METH + Aβ, prevention group), and control (Group 5).

MTT assay for estimating cell viability

Astrocytes were seeded in a 96-well plate (10,000, 15,000, and 20,000 cells per well), fed with 5% FBS in DMEM, and incubated for 24 h. Then, they were exposed to 0.8, 1.6, 3.1, 6.2, 12.5, 25, 50, and 100 μM concentrations of METH (donated by Tehran University), which were dissolved in water and used for 24-, 48-, and 72-h treatment. In addition, MTT (Sigma-Aldrich, USA) (5 mg/ml in PBS) was added. We used DMSO to dissolve the crystals. The measured absorbance was at 570 nm.

Cell treatment

Astrocyte cells were cultured in a 6-well plate (200,000–250,000 cells per well). According to the IC50, METH was added to DMEM containing 10% FBS to reach the final concentration of 12.5 μM. METH exposure for the cell was performed for 24 h [23]. Regarding the treatment with Aβ, 10 μM of Aβ was kept at 37 °C for 72 h (fibril formation) and added to DMEM plus F12 lacking FBS [24], and the cells were exposed to amyloid for 24 h [25]. The related groups included the cells with Aβ (Group 1), METH (Group 2), Aβ + METH (METH after adding Aβ for
24 h: treated group; Group 3), METH + Aβ (Aβ after adding METH for 24 h: prevention group; Group 4), and control.

**RNA extraction**

Total RNA was isolated from astrocyte culture by using an RNA extraction kit (Roche 11828665001) according to the manufacturer’s recommendation. The concentration of RNA samples was determined by measuring optical density at 260 nm. The quality of RNA was confirmed by detecting 18S and 28S bands on agarose gel electrophoresis. The RNA samples were incubated with DNase at room temperature for 15 min to remove residual DNA contamination.

**cDNA synthesis**

The total RNA from each sample was used to generate cDNA with oligo (dT) primers according to the manufacturer’s protocol Thermo (K1621).

**Oligonucleotide primers**

GAPDH was used for housekeeping genes, and primer 3 was applied to design all of the primers. Table 1 indicates the primer sequences.

**Molecular analysis**

**Quantitative real-time PCR analysis**

The GSK3β, Cdk5, PKCa, Bcl-X, and Bax genes related to AD and apoptosis were evaluated by using the housekeeping gene (GAPDH). The primers were previously checked by conventional RT-PCR and agarose gel (1.5%) electrophoresis. Real-time quantitative polymerase chain reaction (PCR) was performed by using Ampliqon PCR Master Mix (A314406) and Qiagen Rotor-Gene Q system. All of the experiments were performed in triplicates. Table 1 indicates the real-time PCR primers specific for the test and GAPDH gene. The cycling protocol consisted of an initial 5-min denaturation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final 5-min extension step at 72 °C. CT relative quantification method was used and the obtained CT values were normalized for endogenous reference [26].

**Western blotting to prepare total protein extraction**

The astrocyte cells were digested in NP40 lysis buffer (CMG-NP40). The cocktail protease and phosphatase inhibitor (Roche 11836153001, 04906845001, respectively) were added to the cell lysate for 30 min at 4 °C. Following centrifugation at 14,000xg, the supernatant was stored at −70 °C. Then, the protein concentration was determined by using a Bradford Assay Protein Kit (Bio Basic SK3031) according to the manufacturer’s protocol [27]. In addition, the proteins were mixed with a loading buffer (Tris–HCl 63 mM, glycerol 30%, SDS 2%, Bromophenol blue 0.05%, pH 6.8), and boiled for 5 min at 95 °C. Further, 10% polyacrylamide gel was used. Accordingly, 80 μg of protein was loaded in each well at that time and the electrophoresis was conducted at 125 mV for 1 h. After gel electrophoresis, all proteins were transported into a PVDF membrane with 320 mA for 2 h at 4 °C (Bio-Rad 1620174), and then blocked with BSA 5% for 1.5 h at room temperature. Membranes were incubated overnight with monoclonal antibody (p-GSK3β-sc-373800) (1:500), monoclonal antibody (GSKα/β-sc-7291) (1:500) at 4 °C, monoclonal (β-Actin (c4) sc-47778) (1:500) at 4 °C and anti-tau (phospho S396 EPR2731) Abcam (1/10,000) at 4 °C. Afterward, the membranes were washed three times for 5 min in Tris-buffer saline solution with 0.1% Tween (TBS/T) incubated with 1:10,000 diluted anti-mouse antibody (Sigma A9044) and 1:2500 diluted goat Anti-rabbit IgG (Elabscience E_AB_1003), respectively. Then, the membranes were washed three times for 5 min in Tris buffer saline solution with 0.1% Tween, and were detected by chemiluminescence. Bands were scanned and the band intensities were calculated by ImageJ. Finally, the bands were normalized to the intensity of β-actin in each sample.

**Apoptosis and necrosis survey**

Briefly, pre-treated astrocyte cells were harvested with trypsin and washed with 0.01 M PBS twice. Along with centrifugation at 2000 rpm for 5 min, the cells were

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**Table 1** Primes used for real-time PCR

| Primer   | Forward                | Reverse                | Length (bp) |
|----------|------------------------|------------------------|-------------|
| GAPDH    | GACCACCTTGTCAAGCTCATTTCC | GTGAGGGTCTCTCTCTCTCTCTTTG | 168         |
| Bax      | TGGAGCTGCAAGGATGATGTTG | GAAGTTGCGCTAGAAACACATG | 98          |
| Bcl-x    | CTGAAATCGGAGATGGGACC   | TGGGATGTCAGTCAGCTGAA   | 211         |
| PKCa     | GGCTCCCAAGAGTGCCATG    | AAGGTGGGGTCTCGTGAAGTG  | 122         |
| Gsk3β    | ACAAACAGTGTTGGCAACTCC  | TTCTGGATGCGGACACATTCT  | 89          |
| CDK5     | GGAAGGCACCTACGGAACTG   | CGGCCACACCCTCATCATCG   | 102         |
re-suspended in 500 μl of binding buffer at the density of 1×10^6 cell/ml, along with adding 5 μl Annexin, V-FITC, and 5 μl PI, ((ANXVF-200T)) respectively. Next, the cells were incubated in the dark at 25 °C for 15 min and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, and USA).

**DNA content cell cycle analysis (propidium iodide)**

The pre-treated astrocyte cells were harvested with trypsin and washed with PBS by centrifugation at 2000 rpm for 5 min at 4 °C. The cells were re-suspended in cold PBS including DNase-free Ranse (Sigma) and stained with Propidium Iodide (PI) containing 1% Triton X-100 (v/v) (Sigma). The solution was incubated at 20 °C for 30 min (protected from light) and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) [28].

**Hoechst staining assay**

The treated astrocyte cells were harvested and washed with PBS. The density of the cell should be 1*10^4 cells/ml in PBS (1:1 v/v). The cells were incubated for 30 min at the room temperature with Hoechst 33342 (Invitrogen, H3570) (5 mg/ml).

**Statistical analysis**

SPSS v16 and GraphPad Prism were used for statistical analysis. Each of the treatment group was compared with Aβ group by using independent sample t-test in real-time PCR. ANOVA followed by Dunnett post hoc test was used to evaluate the results of the western blotting analysis. P < 0.05 was accepted as the level of significance. All error bars in the figures are based on the results of mean ± standard deviation (SD). Each experiment was performed in triplicate.

**Results**

**Effect of methamphetamine on astrocyte viability**

The cells were incubated with different concentrations of METH for 24, 48, and 72 h. The results demonstrated the cellular 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 h. Therefore, 12.5 μM concentration of METH was used for further evaluation (data not shown).
10 µM Aβ for 24 h, cells arrest in G2, and enter S compared to the Aβ group. Regarding the Aβ + METH group, G1 decreases, enters the S phase, and the cells arrest in G2, compared to the Aβ group (Table 3).

**Apoptosis detection with Hoechst staining assay**

In the Hoechst staining, apoptotic cells (fragmented nuclei) are often observed in the Aβ group (Fig. 5).

**Discussion**

Based on the hypothesis, astrocytes respond to the low dose of METH exposure and can affect tau hyperphosphorylation and apoptosis. Thus, it may have a protective effect on AD. The high doses of METH cause dopamine secretion and neurotoxicity. However, the moderate activation of dopamine receptors plays a protective effect on neurons. In addition, a mild dose of METH increases cell survival by reducing apoptotic cell death [29]. Therefore, we used a low dose of METH. The results indicated that a low dose of METH can decrease the amount of apoptosis in the prevention and treatment groups. Also, the effect of a low dose of METH on the expression of PKCα was evaluated. An increase occurred in clearing Aβ while activating the α-secretase plays a role in clearing Aβ and PKCα influence the activity of this enzyme [30]. The results indicated that the effective dose of METH decreases the gene expression of PKCα in the prevention group compared to the experimental model of astrocyte induced with AD, while an increase occurred in the treatment group. Although a highly abundant protein usually has a highly expressed mRNA, some differences may be observed in the amount of protein and mRNA because of the processes between transcription and translation. For example, the difference between protein and mRNA includes the half-life of protein from several minutes to several days, and the half-life of mRNA is shorter than 2–7 h [31]. Thus, it is suggested that the decrease in mRNA expression of PKCα results from its half-life or the presence of miRNA or RNA binding protein which may destroy the pool of mRNA in the cell culture.

In the neuronal damage, Cdk5 and GSK3β are involved in AD leading to tau phosphorylation [32]. In this study, the gene expression of Cdk5 decreased in the prevention group, which increased in the treatment group compared with the AD model. The reduction in the amount of Cdk5 in the prevention group indicates that METH can considerably influence Cdk5 expression. A large body of research indicated that Cdk5 is associated with neurotoxicity and neurodegeneration. Other studies reported that METH exposure increases the level of phosphorylated tau, and down-regulation of Cdk5 can prevent overexpressing phosphorylated tau

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**Fig. 1** The effect of METH on the expression of Bax and Bcl-X genes. The values put onto each graph represent the relative fold change calculated by calibrating the ΔCt data Bax and Bcl-X gene expressions in several experiments. A Bax decreases in group-3 (Aβ + METH) and group-4 (METH + Aβ) in compare to group1 (Aβ) (P = 0.035 and P = 0, respectively). B An increase in Bcl-X is not significant in group-3 (Aβ + METH), and group-4 (METH + Aβ) increases (P = 0.056). C The ratio of Bax/Bclx in group-3 (Aβ + METH) and group-4 (METH + Aβ) was decreases in compare to group1 (Aβ) (P = 0.035). Error bars indicate SD. The level of significance was considered as *P ≤ 0.05. Group-1 (Aβ), group-2 (METH), group-3 (Aβ + METH), group-4 (METH + Aβ)
based on the type of cell line, the concentration of METH, and exposure time (SH-SY5y, 0.5–2 mM, 0–24 h, respectively) [33]. However, the results of the present study indicated that the cell line is astrocyte, exposure time is 24 h, and the concentration of METH is lower since it was designed for treating infected cells.

The stimulation of cells with a protein kinase B (AKT) can make the GSK-3 kinases inactive by direct phosphorylation of an inhibitory residue at S21 in GSK-3α or S9 GSK-3β [34]. GSK-3β is the essential kinase which participates in the phosphorylation of tau, which could increase hyperphosphorylation of tau during the intracellular aggregation of Aβ. Further, the activation of GSK-3β-mediated tau phosphorylation in AD reduces its affinity to microtubules, and the inhibition of GSK-3β can significantly reduce tau phosphorylation [35]. In the present study, METH causes to reduce the protein expression of GSK-3α and 3β as their dephosphorylated and activated form compared to Aβ group. Thus, hyperphosphorylated tau protein decreased in the presence of METH. Additionally, the protein expression of GSK-3β as their phosphorylated and inactivated form increases in the presence of METH compared to Aβ group. Thus, hyperphosphorylation of tau as residue ser396 decreases in the prevention (METH + Aβ) and treatment groups (Aβ + METH).

It is worth noting that METH had pretreatment and treatment effect on the astrocytes infected with Aβ since Bcl-X increased significantly in both groups. Regarding the Bcl-X gene expression, METH and estrogen have a similar effect, both of which increase Bcl-X due to the anti-apoptotic effect of the two-mentioned reagents. The results of the previous study indicated that the low concentration of METH without any effect on glucose uptake increases the expression of glucose transporter protein-1.
Fig. 3  A Gel mobility of protein expression. The effect of METH on the expression of B GSK3α, C GSK3β, D p-GSK3β-ser9, and E p-tau-ser396. All of them decreased in group-3 (Aβ+METH) but GSK3α, GSK3β, and p-tau-ser396 decreased in group-4 (METH+Aβ) compared to group-1 (Aβ), while p-GSK3β-ser9 increased in group-4 (METH+Aβ) compared to group-1. The numbers below the western blot including the following groups: 1 (Aβ), 2 (METH), 3 (Aβ+METH), 4 (METH+Aβ), 5 (control).
(GLUT1) in primary human brain endothelial cell (hBEC), while a high concentration of METH decreases the glucose uptake and GLUT1 protein level in hBEC culture [36]. Further, increased GLUT1 can improve the insulin signaling pathway since AD was identified as type 3 diabetes [37], increased glycolysis, and GLUT1, which are correlated with the inactivation of GSK3β or GSK3β-Ser9 phosphorylation which controls the production of ROS.

Table 2  Apoptosis and necrosis analysis by flow cytometry

| Group       | Aβ   | METH | Aβ + METH | METH + Aβ | Control |
|-------------|------|------|-----------|-----------|---------|
| Live cell   | 89   | 92.4 | 90.3      | 91.5      | 95.3    |
| Late apoptosis | 2.02 | 2.06 | 2.47      | 2.45      | 1.22    |
| Early apoptosis | 4.13 | 3.91 | 2.33      | 2.93      | 1.05    |
| Early & late apoptosis | 6.15 | 5.97 | 4.8       | 5.38      | 2.27    |
| Necrosis    | 4.87 | 1.62 | 4.95      | 3.13      | 2.45    |

Fig. 4 The effect of METH on the apoptosis in astrocyte cells. the control group shows the untreated astrocyte cells after 72 h, (Aβ) group treated with 10 µM Aβ for 24 h, (METH) group after astrocyte was treated with 12.5 µM METH for 24 h, (Aβ + METH) group with flow cytometry carried out on astrocyte which was treated for 24 h with Aβ and then 24 h with METH, (METH + Aβ) group that astrocyte was treated with METH for 24 h and after that treated with Aβ for 24 h, and then flow cytometry 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)

Table 3  Cell cycle arrest in different treated groups

| Group       | 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 addition, we decided to use a low dose of METH because of its effect on the GLUT1 and AD.

During stress conditions, neurons for protecting the cells from harmful factors reactivate their cell cycle and the cell cycle is stopped in the G2 phase [39]. In AD, the neuron cells in areas of the brain are affected by the disease, the G1/S checkpoint often becomes dysregulated, and the prior neurons start the replication of DNA to reach a G2/M phase. Unlike the G1/S checkpoint, the G2/M checkpoint works properly, which prevents dividing neurons [40]. AD neurons are often arrested in the G2 phase. In the present study, G1, G2, and S were evaluated in all of the treatments. Finally, the cells may go to autophagy. In other words, the cells remain for a short time in the G2 phase, leading to an increase in the G1 phase and an increase in G2 in all of the groups except in the treatment group.

It is better to co-culture astrocytes and neurons together and evaluate cell signaling pathway, although it was not considered in this study due to the lack of time and cost. Thus, it is recommended to consider the protein expression pathways of AKT, CDK5, CDK1, CDK2, and molecular pathway of apoptosis simultaneously with flow cytometry in further studies. Due to the discrepancy in the expression of pkcα, the expression of pκcα protein should be evaluated in the future.

Conclusion

In conclusion, since astrocytes as the main supportive cells in CNS and take part in homeostasis, under physiological or pathological conditions, we used a low dose of METH on reactive astrocytes and evaluated the main signaling pathway in AD. Low-dose of METH reduced Early & late apoptosis and increased live cell. It also increased and decreased Bax and Bcl-X gene expression, respectively, and also decreased the gene and protein expression of GSK as a marker of AD. The findings indicated the beneficial use of a low dose of METH in managing AD. Thus, it is suggested that the used dose can be considered as a new potential therapeutic method.

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Author contributions Investigation: BS; Conducted the experiments and Writing-review & editing: BS and MDS; Data analysis: BS, MDS and GHR; Supervision: MDS, NM, and GHR; and All authors have read and approved the manuscript before submission.

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Declarations

Conflict of interest  The authors declare that they have no conflict of interest.

Ethical approval  All samples used in the study were collected in accordance with the guidelines approved by the ethics committee of our University with approval ID: IR.IAU.DAMGHAN.REC.1398.005. The parents who donated the foetal tissue gave their consent for the use of the foetal tissue in this study.

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