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ORIGINAL ARTICLE

What Happens When Methamphetamine Is Added to Nutrients of Culture Medium: *In Vitro* Effects on Proliferation, Differentiation and Apoptosis of Human Fibroblast Cells

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ABSTRACT

Background: Methamphetamine use can provide a quick and pleasurable rush, increased energy, heightened attention, and euphoria, but may also result in many adverse effects. Various cell lines have been utilized *in vitro* to determine the adverse impacts of methamphetamine on those cells. As enough data are not available on the effect of methamphetamine on skin fibroblast cells, this study determined its *in vitro* effect on proliferation, differentiation and apoptosis of human skin fibroblast cells.

Methods: Phenotypic characteristics of fibroblasts cells were determined and real-time PCR examined the expression of matrix metalloproteinase1 (MMP1), matrix metalloproteinase 3 (MMP3), integrin alpha 11 (ITGA11), CD106, CD10, and CD26 markers. MTT assay checked the toxicity of recreational doses of 6 and 60 µM of methamphetamine. Quantitative real time polymerase chain reaction (qPCR) assessed expression of Bax, Bcl-2 and PPARγ genes.

Results: Fibroblast cells were morphologically spindle-shape and were positive for fibroblast markers of CD10, CD26, MMP1 and MMP3 and negative for mesenchymal markers of ITGA11 and CD106. MTT assay revealed a decline in proliferation of fibroblast cells when they were exposed to methamphetamine. The expression of Bax and PPARγ genes increased and decreased for Bcl-2 gene after exposure of cells to methamphetamine.

Conclusion: Our results confirmed adverse effects of methamphetamine on proliferation, viability and differentiation of skin fibroblast cells revealing a reduction in cell proliferation and differentiation as well as an increase in cell apoptosis. These findings can open a window to health status of people who target methamphetamine use for recreational purposes.

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Introduction

Amphetamine-type stimulants are considered a global popular street substance with relatively low cost and they are highly addictive. Among them, methamphetamine (most commonly known as "meth," "crystal meth," "tina," "speed," "crank," "chalk," and "ice,") accounts for 72% of amphetamine-type stimulants (1). Methamphetamine is a psychostimulant substance that affects central nervous system (CNS) via a non-exocytotic mechanism that leads to the release of monoamine neurotransmitters, such as dopamine, norepinephrine and serotonin (2). Its use can provide a quick and pleasurable rush followed by increased energy, heightened attention, and euphoria, but may also result in many adverse effects such as seizures, stroke, decreased appetite, increased respiration, increased heart rate, heart attack, cardiomyopathy, psychosis, rhabdomyolysis, impaired physical appearance, stimulant dependence, overdose, and death. Among women, its use can lead to intrauterine growth retardation, placental abruption, and preterm birth, and adverse consequences in their children (1, 3).

Methamphetamine abuse can be accompanied by oral diseases, tooth decay and gingival abnormalities (4). Methamphetamine absorption happens via skin lipids and can cause several adverse effects (5). *In vivo* studies have shown its adverse effects when it is used for weight loss purposes (6). Mesenchymal stem cells (MSCs) have been utilized for *in vitro* effects of methamphetamine on various cell lines (7). MSCs have opened a new door in regenerative medicine because they are easily expanded and possess various healing and aesthetic functions (8). These cells are non-hematopoietic and are plastic-adherent, fibroblast shape with self-renewing, differentiation and migration activities (9). They express mesenchymal surface markers and lack expression of hematopoietic markers (10). MSCs can be isolated from various sources including adipose tissue (11), bone marrow (12), dental pulp (13), Wharton's jelly (14) and menstrual blood (15). As enough data are not available on the effect of methamphetamine on skin fibroblast cells, while methamphetamine can penetrate into and through the human skin following exposure, the aim of this study was to determine its *in vitro* effects of methamphtamine on proliferation, differentiation and apoptosis of human skin fibroblast cells.

Materials and Methods

Fibroblast cells were provided and utilized from a previous work described before (16); while the research protocol was verified by Kazeroon Islamic Azad University Ethics Committee. In brief, human foreskin samples were obtained from surgical procedures after obtaining informed consent and they were used to isolate skin fibroblast cells. The obtained skin samples were transferred to the laboratory in falcon tubes containing complete DMEM-F12 supplemented with 10% fetal bovine serum (FBS, Gibco, USA) on ice. They media were removed and the tissue samples were prepared for cell culture. The skin samples were first washed 3 times with phosphate buffered saline (PBS, Biowest, USA) supplemented with 1% penicillin/streptomycin (Thermo Fisher Scientific, USA). Then, the washed samples were cut into small pieces employing a sterile blade. They were later transferred to a falcon tube containing 0.5% dispase (Gibco, USA) at 4°C for 12 h. The epidermis was removed and the remained dermis was cut into small pieces and treated with 0.1% collagenase I (Sigma Aldrich, USA) at 37°C for 4 hours. To inactivate the enzyme effect, DMEM-F12 supplemented with 10% FBS was added, followed by filtering of the samples through a 70 µm cell strainer.

The filtered product was centrifuged at 100 g for 10 min, the upper media was removed and the precipitate part was suspended in 1 mL of DMEM-F12 supplemented with 10% FBS and 1% penicillin/streptomycin. Cell suspension was seeded in T25 tissue culture flasks containing 4 mL of the complete media and finally put in incubator with 5% CO_2 with saturated humidity at 37°C. The media were changed and replaced every three days to reach 80% confluence. To subculture the fibroblast cells, the cells were treated with 0.025% 1x Trypsin–EDTA (Sigma-Aldrich, USA).

Characterization of skin fibroblast cells were provided and used from a previous research explained before (16). Phenotypic characteristics of fibroblasts cells were provided from our previous work too, while cells were examined under an invert microscope (Nikon, Japan) and real-time PCR was employed to determine the expression of matrix metalloproteinase1 (MMP1), matrix metalloproteinase 3 (MMP3), integrin alpha 11 (ITGA11), CD106, CD10, and CD26 markers that should be positive for fibroblast markers of CD10, CD26, MMP1 and MMP3 and negative for mesenchymal markers of ITGA11 and CD106 in human skin fibroblast cells (16).

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay was employed to check the toxicity of recreational doses of 6 and 60 µM of methamphetamine (Sigma-Aldrich, USA) as described in several studies (7, 17, 18).

Methamphetamine was dissolved in PBS and was added to each well in 0.1 mM PBS to the cells twice with a 6 hours interval, while the control cells were not exposed to methamphetamine and just treated with culture medium. They were incubated at 37°C for 4 hours and then were enumerated for 7 days, while 3×10^4 cells at 3rd passage/per well were transferred into 5 wells of 12-well culture plates. One well was used as control and 4 as methamphetamine-treated cells. All assays were repeated in triplicate.

Quantitative real time polymerase chain reaction (qPCR) was used to assess expression of Bax, Bcl-2 and PPARγ genes. After removing the culture medium, washing the cells with PBS, treatment with trypsin/EDTA addition of culture medium, centrifugation (5 min, 1200 rpm), removing the supernatant, the sediment cells were used for real time PCR. First, RNA Extraction was conducted utilizing an RNA extraction kit (Cinna Gen Inc., Tehran, Iran). Then the extracted RNA was examined at optical density ratio of A260/A280 and A260/A230 using a Nanodrop spectrophotometer (Nanodrop; Thermo Fisher Scientific,Waltham, USA). cDNA was further prepared utilizing 1000 ng total RNA by applying the Revert Aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Page 11/14 USA).

GAPDH was considered as an internal control gene. The gene sequences were determined employing NCBI database and primer sets were designed by primer3 software (Table 1). SYBR Green I was defined as reporter dye to conduct Step One Real-Time PCR reactions (Applied Biosystems, Waltham, USA). In each reaction, 200 nM of each primer was added to target the specific sequence. The PCR condition was set at 94°C for 10 min followed by 40 cycles at 94°C for 15 s, at 60°C for 60 s, and melting curve analysis ramping from 65 to 95°C. The amplification signals of different samples were normalized to B2m cycle threshold (Ct), and then the 2- DDCt method was applied to compare mRNA levels of various groups, which represented a fold-change in data analysis.

The PCR efficiency percentage for the desired primers was presented in Table 2. Data for GAPDH and Bax genes were presented in Figure 1 revealing fragment duplication (Figure 1A), melting curve (Figure 1B), and linear slope (Figure 1C) for GAPDH gene, and fragment duplication (Figure 1D), melting curve (Figure 1E), and linear slope (Figure 1F) for Bax gene. Figure 2 displays findings for Bcl-2 and PPARγ genes for fragment duplication (Figure 2A), melting curve (Figure 2B), and linear slope (Figure 2C) of Bcl-2 gene and melting curve (Figure 2D), fragment duplication (Figure 2E), and linear slope (Figure 2F) of PPARγ gene.

Results

Fibroblast cells were morphologically spindle-shape (Figure 3). Employing real-time PCR, the cells were positive for fibroblast markers of CD10, CD26, MMP1 and MMP3 and negative for mesenchymal markers of ITGA11 and CD106 (Figure 4) (16).

MTT assay revealed a decline in proliferation of skin fibroblast cells until day 7 days when they were exposed to 60 µM of methamphetamine in comparison to control. An increase in cell proliferation happened on day 5th that may be due to media change on that day (Figure 5). On days 4, 6 and 7; the reduction in cell proliferation was

Table 1: Sequence of reverse primers of desired genes for Real-time PCR reaction.

Genes	Primer Sequences	Sizes (bp)
Bax	Forward: 5'-GCCCTTTTGCTTCAGGGTTTCA -3'	108
	Reverse: 5'-CAGCTTCTTGGTGGACGCAT-3'	
Bcl-2	Forward: 5'-ACGAGTGGGATGCGGGAGATGTG-3'	245
	Reverse: 5'-GCGGTAGCGGCGGGAGAAGTC-3'	
PPARγ	Forward: 5'-CTATGGAGTTCATGCTTGT-3'	177
	Reverse: 5'-CTGATGGCATTATGAGACA-3'	
GAPDH	Forward: 5'-GGCTGTTGTCATACTTCTCATG-3'	207
	Reverse: 5'-CCATCTTCCAGGAGCGAGA-3'	

bp: base pair

Table 2: The PCR efficiency percentage of the desired primers.

Gene	Slope of linear concentration (M)	Coefficient of determination (r^2)	PCR efficiency (%)	Melting temperature (Tm)
GAPDH	3.275	0.983	101	(60) 84.7
Bax	3.260	0.984	102	86.5 (60)
Bcl-2	3.452	0.993	94	85.4 (60)
PPARγ	3.277	0.998	101	(60) 79.4

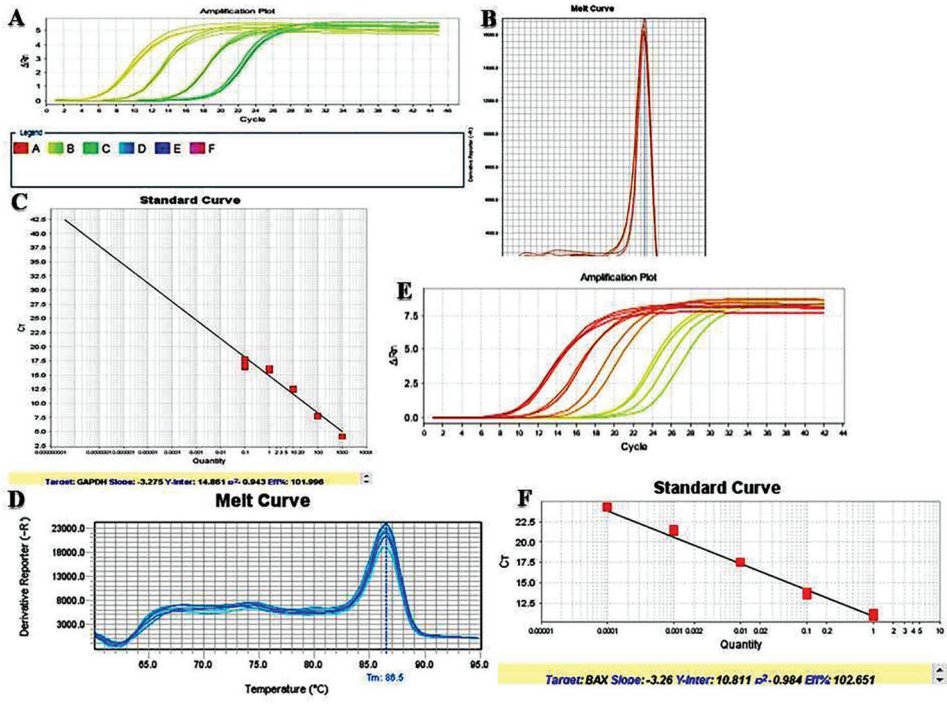

Figure 1: Data for GAPDH and Bax genes. GAPDH gene: **A.** Fragment duplication, **B.** Melting curve, **C.** Linear slope (Figure 1C). Bax gene: A. Fragment duplication, B. Melting curve, C. Linear slope.

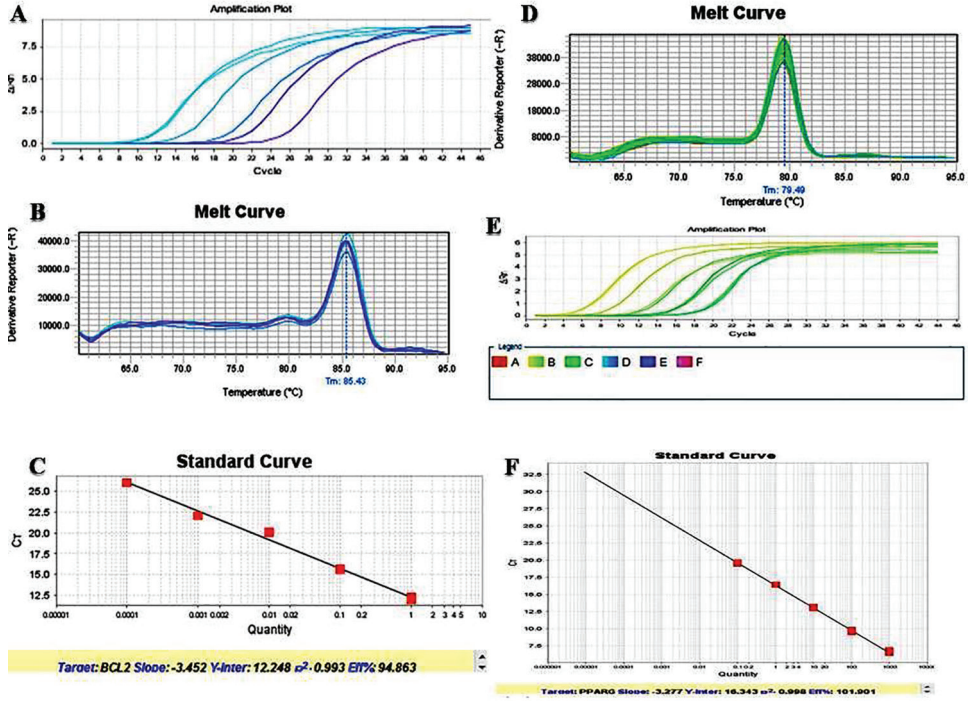

Figure 2: Findings for Bcl-2 and PPARγ genes. Bcl-2 gene: **A.** Fragment duplication,C. Linear slope.

statistically significant (p=0.01) revealing the toxic effect of methamphetamine on proliferation and viability of fibroblast cells.

The expression of apoptotic gene of Bax increased and Bcl-2 decreased after exposure of cells to 6 and 60 µM of methamphetamine. Bax gene expression increased 6.38 and 7.25 times compared to the control group, respectively (p=0.0001). The expression of Bcl-2 gene decreased after exposure to methamphetamine compared to the control group (p=0.02). The PPARγ gene expression for fibroblast cells exposed to methamphetamine increased by 2.74 and 1.50, respectively (p=0.01, Figure 6).

Discussion

National Institute of Drug Abuse (NIDA) has reported the immediate effects of acute methaphetamine use (i.e., active intoxication) as confidence, euphoria, and stimulant-like effects (e.g. hyper-focus, high energy) with

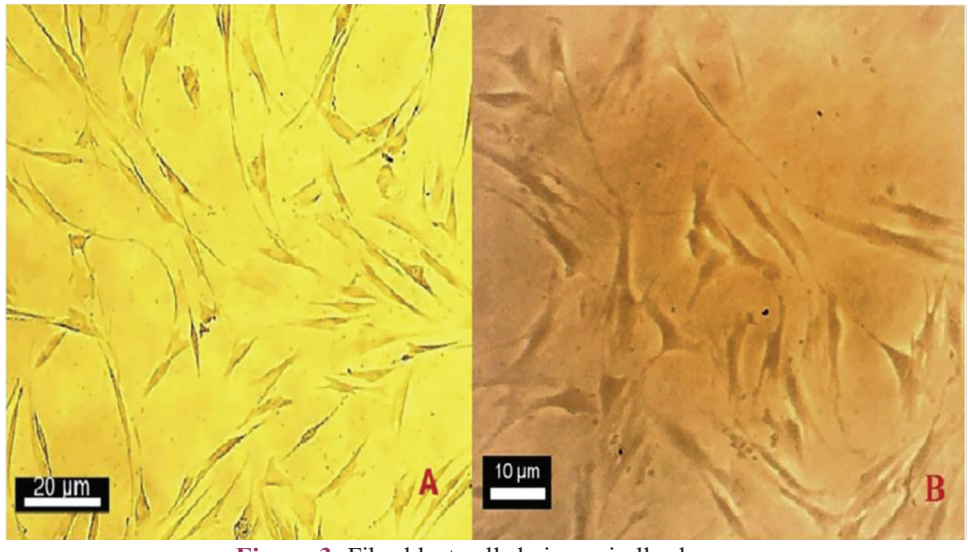

Figure 3: Fibroblast cells being spindle shape.

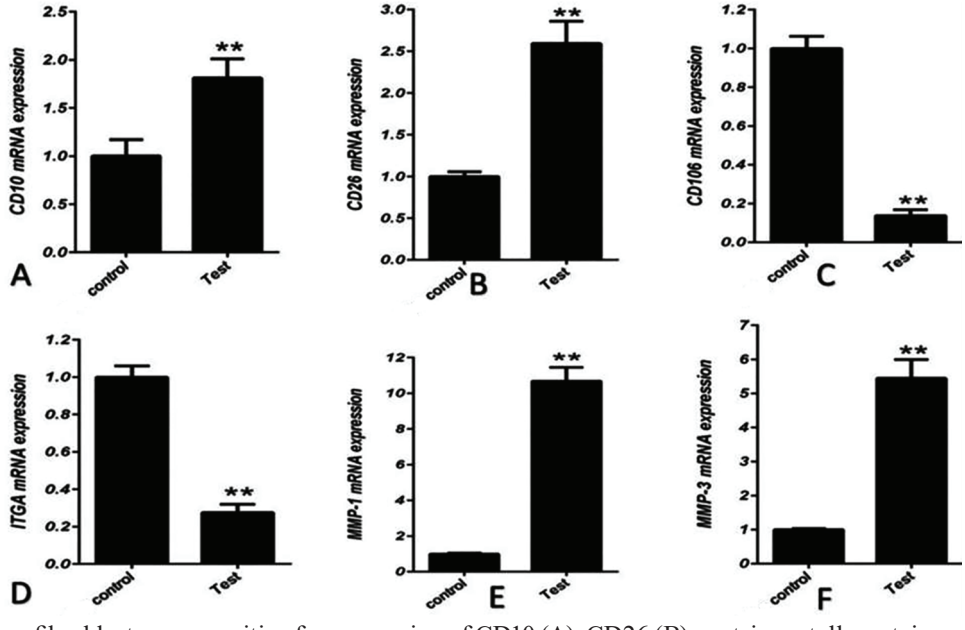

Figure 4: Human fibroblasts were positive for expression of CD10 (A), CD26 (B), matrix metalloproteinase1 (MMP1) (E), and matrix metalloproteinase 3 (MMP3) (F), and negative for expression of CD106 (C) and integrin alpha 11 (ITGA11) (D).

complications such as low appetite, nausea, difficulties sleeping, hypothermia, and heightened physiological reactions (e.g., heart rate) in heavier consumptions (19). Chronic (i.e., habitual) of methamphetamine utilization were demonstrated with complications such as memory loss, deficits in cognitive and motor skills, concentration difficulties, mood disturbances, violent behavior, psychosis, and several physiological issues (e.g., skin sores, weight loss, dental problems) (20). It was shown that methamphetamine can penetrate into and through the human skin following exposure, while smooth and dense surfaces favor methamphetamine retention and transfer and when the pH exceeds 4, methamphetamine is converted to its volatile freebase form that reminds the public health importance of methamphetamine use (21).

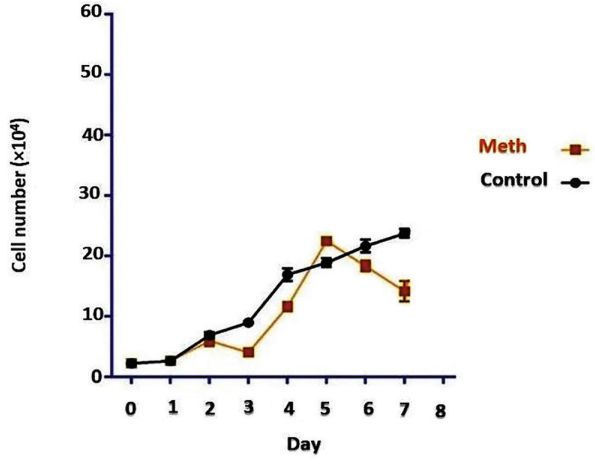

Figure 5: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay of skin fibroblast cells exposed to 60 µM of methamphetamine in comparison to control treated with just cell culture media (p=0.01 on days 4th, 6th and 7th).

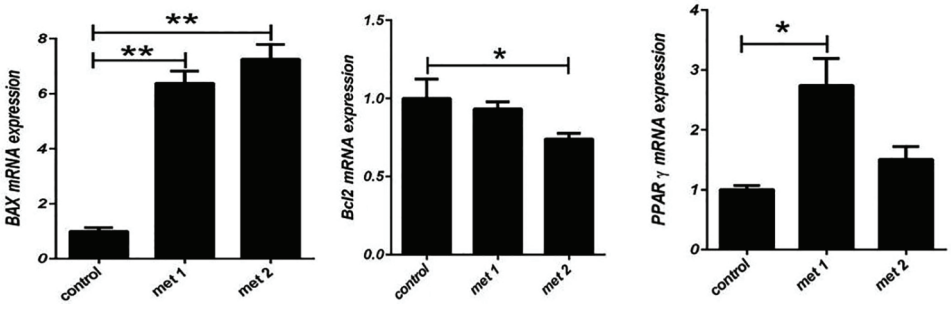

Figure 6: The gene expression of apoptotic genes of Bax and Bcl-2 and PPARγ gene when skin fibroblast cells were exposed to doses of 6 and 60 µM of methamphetamine. Bax gene expression increased 6.38 and 7.25 times, respectively when compared to the control grou, (p=0.0001 for Bax gene, p=0.02 for Bcl-2 gene and p=0.01 for PPARγ gene).

There are several studies investigating the *in vitro* effect of methamphetamine on different body cells. Mohammadzadeh *et al.* reported the negative influence of recreational dose of methamphetamine on Wharton's jelly stem cells (WJSCs) to reduce the proliferation and viability of these cells (7). Anari *et al.* and Jaafary-sheybani *et al.* exposed adipose tissue derived stem cells (AdSCs) to methamphetamine and observed an increase in apoptosis of stem cells and a reduction in proliferation and viability of these cells (18, 22). Zamani-Pereshkaft *et al.* illustrated the negative effects of methamphetamine on SH-SY5Y cell proliferation, differentiation and expression of KI-67, PPARα, PPARγ and Bax genes (23, 24). It was shown that methamphetamine can negatively affect the proliferation of neuron progenitor stem cells in the dentate gyrus area of the brain (25). Goudarzi *et al.* displayed a significant reduction in proliferation of endometrial stem cells and an increased apoptosis when MSCs were treated with methamphetamine (26-28). The impact of methamphetamine has also been evaluated on gingival fibroblasts revealing an increase in activity/activation of pro-matrix metalloproteinases 2 (MMP-2) (29). These findings are in agreement with our results for adverse effects of methamphetamine on MSCs leading to a decrease in cell proliferation and viability and a modification in expression of several genes.

It seems that fibroblast cells can be a suitable model in cellular and developmental biology, and pharmaceutical investigations. Fibroblast cells have been characterized in many animals for *in vitro* studies (30-32); as well as human fibroblast cells (16). As methamphetamine accelerates cellular senescence and activates the transcription of genes involved in inflammation and aging through a mechanism that requires increased ceramide production, it can induce apoptosis and a reduction in cell proliferation (33). In mice, methamphetamine was shown to change cell karyotype (34).

Methamphetamine causes a delay in the cell cycle when passing from the G0/G1 phase to the S phase, which is related to the decrease in the expression of cyclin E, and pEGFR, pERK1/2 proteins. Cyclin E protein together with cyclin-dependent protein kinase 2 (Cdk2) form a complex that is involved in the progression of the G1 phase and the initiation of DNA replication in the S phase (25). Huang *et al.* showed that caspase 11 plays an essential role in methamphetamine-induced apoptosis of dopaminergic neurons, and they also showed that exposure to methamphetamine increases the expression of caspase 11 both *in vitro* and *in vivo* (35). In vitro effect of methamphetamine depends on both the duration of exposure and the dose of methamphetamine. It was shown that methamphetamine can increase the expression of the anti-apoptotic protein Bcl2 and decreases the amount of the pro-apoptotic protein Bax in the ventral hippocampus region of the brain (36). Cadet and colleagues found that the sigma 1 receptor had a significant effect on the activation of cell death when microglial cells were treated with methamphetamine and induced apoptosis (37). Tokunaga *et al.* evaluated the pre-oxidative DNA damages of methamphetamine in different regions of the brain and demonstrated apoptosis in these cells (37). Cai *et al.* in their study reported an increase in the expression of pro-apoptotic genes of BAX, BAD, BAK, BID and a decrease in the expression of apoptosis-inhibiting genes of Bcl-2, Bcl-W, Bcl-Xl after exposure to methamphetamine (38). Cai D *et al.* illustrated that methamphetamine causes apoptosis in vascular endothelial and cardiac microvascular endothelial cells through the Nupr1-Chop/P53-PUMA/Beclin 1 signaling pathway (38). Nudmamud-Thanio and Thanoi revealed that methamphetamine causes apoptosis of the sperm cells in the testis of male rats (39). These cellular changes happen due to a reduction in DNA production, in the number of dividing cells and stimulation of the transcription genes that regulate cell cycle proteins including P53, P21 and pro-inflammatory cytokines that participate in the aging process too (39). Changes

in PPARγ gene expression in nerve cells treated with methamphetamine has also been reported by (40). These findings describe the *in vitro* adverse influences of methamphetamine on proliferation, viability and differentiation of various cell lines. Preparation of tissue samples can be considered as a limitation of our study. They should be provided from aesthetic centers with official written consent of patients and to be healthy and just because of aesthetic surgeries.

Conclusion

Our results confirmed adverse effects of methamphetamine on proliferation, viability and differentiation of skin fibroblast cells revealing a reduction in cell proliferation and differentiation as well as an increase in cell apoptosis. These findings can open a window to health status of people who target methamphetamine use for recreational purposes.

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Authors' Contribution

MZ: Cell culture, MTT assay, data collection; SN: Data analysis; SZ: Manuscript draft preparation; DM: Cell characterization, Manuscript preparation and editing; SZ: Cell culture, MTT assay; IJ: Real time PCR; MH, MN: Skin preparation and cell isolation; FKB: Cell characterization, Manuscript editing and finalizing.

Conflict of Interest

None declared.

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