International Journal of Nutrition Sciences

Journal Home Page: ijns.sums.ac.ir

ORIGINAL ARTICLE

What Happens When Methamphetamine Is Added to Nutrients of Cell Culture Medium? *In Vitro* Assessment of Morphological, Growth and Differential Potential of Wharton's Jelly Stem Cells

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ARTICLE INFO

Keywords:
Methamphetamine
Wharton's jelly stem cells
Morphology
Growth
Differentiation

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Received: July 10, 2022
Revised: October 5, 2022
Accepted: October 16, 2022

ABSTRACT

Background: Methamphetamine use and misuse cause severe side effects in different body organs and are associated with socioeconomic consequences. This study aimed to investigate the *in vitro* effect of methamphetamine on cell morphology, growth and differentiation potential in Wharton's jelly stem cells (WJSCs) when added to the culture media.

Methods: MTT assay was applied to assess the toxicity of recreational dose of 60 µg/mL of methamphetamine on WJSCs. The cells were characterized morphologically, by osteo- and adipo-genic differentiation potential and by flow cytometry. Population doubling time of stem cells was determined and after exposure to methamphetamine was compared with the control.

Results: WJSCs were adherent to culture flasks, were spindle shape and positive for osteo- and adipo-genic inductions and expressed mesenchymal markers, while lacking hematopoietic markers. Methamphetamine resulted in a reduction in cell proliferation and viability.

Conclusion: Our results can be the first study evaluating the *in vitro* effect of methamphetamine on WJSCs at cellular level revealing a decrease in cell proliferation, and viability when cells were exposed and treated with methamphetamine. These findings can be added to the literature especially when methamphetamine is targeted for recreational purposes.

Please cite this article as: Mohammadzadeh N, Mehrabani D, Zare S, Masoumi SS, Rasouli-Nia A, Karimi-Busheri F. What Happens When Methamphetamine Is Added to Nutrients of Cell Culture Medium? *In Vitro* Assessment of Morphological, Growth and Differential Potential of Wharton's Jelly Stem Cells. Int J Nutr Sci. 2022;7(4):233-240. doi: 10.30476/ijns.2022.97432.1210.

Introduction

Psychoactive substances that were initially obtained from plants and animals have a long history of being utilized to change consciousness and to approach the divinities. These substances have recently been prepared by chemical synthesis for recreational use, and have led to drug abuse-related disorders. They can modulate certain neurochemical pathways, and may have beneficial effects on some psychiatric disorders such as post-traumatic stress disease and treatment-resistant depression making these substances an alternative to conventional medicines too. The irruption of new psychoactive substances during the last decade, and despite their recreational and illicit uses, has enlarged the library of substances with potential use on these disorders (1).

At present, many studies have reported the individual effects of opioids and methamphetamine, while millions of people suffer from substance use disorders of methamphetamine. Amphetamine is a derivative of 'α-methylphenethylamine', an older description of methylamphetamine, metamphetamine, and N-methyl-1-phenylpropan-2-amine that is N-methyl derivative. Methamphetamine is a highly addictive psychostimulant drug having a significant abuse potential inducing neurotoxic effects especially on brain (2). Brain regions such as the infralimbic cortex, cingulate cortex, nucleus accumbens, dorsal striatum, central amygdala, and basolateral have been shown to play important roles in addiction-related behavioral alterations (3). Methamphetamine use and misuse are also associated with severe socioeconomic consequences. The users may develop tolerance, lose control over drug taking behaviors, and suffer frequent relapses even during treatment, while the consequences were reported in all ages, sexes, and races (4).

Therefore, methamphetamine use, manufacturing, and the resulting contamination are considered a significant issue impacting the environment, the economy, and the public health. It has adverse health risks for individuals and first responders with exposures from the inhalation of airborne residues or from contact with contaminated substances (5). The neurotoxic effects of methamphetamine are via the mitochondrial functional impairment, oxidative stress, the activation of astrocytes and microglial cells, endoplasmic reticulum stress, axonal transport barriers, apoptosis, and autophagy. Methamphetamine abuse was shown to increase the chances of developing neurotoxic disorders such as Alzheimer's disease (AD), and Parkinson's disease (PD) through increasing the expression of alpha-synuclein (ASYN) with subsequent neuro-degeneration due to biological variations in the brain region or genetic and epigenetic differences (6).

Methamphetamine abuse is also associated with adverse effects on the cardiovascular system including malignant and benign arrhythmias, coronary vasospasm, dilated cardiomyopathy, and atherosclerotic coronary artery disease, while the mechanisms for the cardiotoxicity are through Sigma-1 receptors and trace amine-associated receptor 1 (TAAR1) leading to a myriad of negative downstream effects including mitochondrial dysfunction, increased reactive oxygenating species (ROS), and modulations of intracellular calcium and are mediated by an increase in circulating catecholamines too (7). Methamphetamine use is now recognized as a definite cause of pulmonary arterial hypertension via reduced expression of carboxylesterase 1 (8). In women, it was demonstrated to induce uterine cysts (9) emphasizing the significant effect of intrauterine methamphetamine exposure across multiple areas of child development and its public health importance (10).

Cessation of methamphetamine use can lead to a significant withdrawal syndrome (11) and methamphetamine addiction is one of the leading causes of mortality, while treatment choices are limited. There are no medications that the Food and Drug Administration verifies for stimulant use disorder. Off-label use of treatment options for stimulant misuse includes anxiolytics, antidepressants, and milder stimulants as replacement agents. Based on shortcomings of the efforts for treatment of complicated psychiatric disorders, recent attention has been toward oxytocin therapy as a possible therapy in the context of social stress, social anxiety, social cognition, and psychosis. Oxytocin produces enhanced connectivity between cortical areas and may alleviate intense withdrawal symptoms (12).

The *in vitro* impact of many substances on mesenchymal stem cells has been investigated including human adipose tissue stem cells (13-15) and bone marrow stem cells (16). Mesenchymal stem cells have been derived from various tissues including adipose tissue (17), bone marrow (18), amniotic fluid (19), and Wharton's jelly (20). They have anti-inflammatory and immunomodulating characteristics (21). They are easily cultivated, have positive expression for mesenchymal markers and are negative for hematopoietic markers (22). Wharton Jelly isolated from mesenchymal stem cells have been described as the best source of mesenchymal stem cells (23). The findings clearly show that Wharton's jelly stem cells (WJSCs) can be the best suggestion for clinical use due to its advantages such

as higher proliferation and differentiation potential, easy access, easy and noninvasive separation, a large number of cells, and no ethical problems (24). As WJSCs are a good candidate for cellular therapy in regenerative medicine (25), this study aimed to investigate the effect of methamphetamine on WJSCs' morphology, growth and differential potential.

Materials and Methods

This study was performed according to the good clinical practices recommended by the Declaration of Helsinki and its amendments. Shiraz Islamic Azad University ethical approval was obtained in addition to written informed consent for human umbilical cord samples provided from Obstetrics and Gynecology hospitals of Shiraz University of Medical Sciences, Shiraz, Iran. Human umbilical cord was the source to isolate WJSCs. Methamphetamine was a donation by Shiraz Police Department for Research Purposes.

The provided umbilical cord tissue was put in a falcon tube containing phosphate-buffered saline (PBS: Sigma-Aldrich, USA), amphotericin B, penicillin, and streptomycin (0.25 µg/mL, 100 U/mL, and 100 µg/mL, respectively (Invitrogen, Thermo Fisher Scientific, USA) and were aseptically taken to stem cell laboratory. They were washed three times with Hanks' balanced salt solution (Invitrogen, Thermo Fisher Scientific, USA) and cut into small pieces of 1 cm and then transferred into a falcon tube containing hyaluronidase (Sigma-Aldrich, USA), collagenase type I and collagenase type IV (Invitrogen, Thermo Fisher Scientific, USA) for enzymatic digestion. They were further placed in an incubator with 5% CO_2 at 37°C and saturated humidity for 45 minutes. In aseptic conditions under a laminar flow hood, the gelatinous mass was filtered by an 18-gauge needle syringe to release cells from the gelatinous mass.

The filtrate was spinned at 200 g for 7 min, the supernatant was discarded and the remained cell pellet was resuspended in 1 mL of culture medium containing 90% Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Invitrogen, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA), 1% penicillin, streptomycin and amphotericin B, 2 mmol/L L-glutamine (Invitrogen, Thermo Fisher Scientific, USA), and 1% nonessential amino acids (Invitrogen, Thermo Fisher Scientific, USA). Cell suspension was transferred into a culture flask already contained 4 mL of DMEM/F12 with FBS, non-essential amino acids, L-glutamine and penicillin, streptomycin and amphotericin B. The viable cells were transferred to a 100-mm cell culture dish at a density of 10^5 cells/cm^2. The culture dishes were put in an incubator with 5% CO_2 at 37 °C and saturated humidity. Medium change was taken place every 3 days. Cell counting was conducted utilizing trypan blue dye and a Neobar hemocytometer (20).

Cells were characterized by morphology, by osteo- and adipo-genic inductions and by flow cytometry too. Morphological characterization of the WJSCs were undertaken to assess being spindle shaped by an invert microscope (20). To determine the osteogenic differentiation of the WJSCs, 5×10^4 cells were counted and transferred into a 6-well plate. When cells were 80% confluent, the medium was altered to osteogenic medium having complete culture medium supplemented with 100 nM dexamethasone (Sigma-Aldrich, USA), 10 mM glycerol 3-phosohate (Merck, Germany), 50 µM ascorbic acid (Merck, Germany) and 15% FBS for 21 days. Media change happened every 3 days and after 21 days, WJSCs were fixed in buffered formalin solution for 20 min, and after three washes with deionized water, the cells were stained using alizarin red dye dissolved in deionized water at pH 4.1 (Sigma-Aldrich, USA). Differentiation was evaluated using alizarin red staining that is bound to calcium mineralized deposits in red color (20).

To characterize adipogenic induction, 5×10^4 WJSCs were seeded in a 6-well plate with cell culture medium. When the cells reached 80% confluence, media change occurred by addition of adipogenic medium consisted of complete culture medium supplemented with 100 nM dexamethasone, 200 µM indomethacin (Sigma-Aldrich, USA), 100 µM ascorbic acid and 15% FBS for 21 days. Buffered formalin solution was used for 20 min to fix WJSCs and after three times washing with deionized water, 0.5% Oil Red O dye (Sigma-Aldrich, USA) dissolved in 2-propanol solution (Merck, Germany) was added for 2 h to stain differentiated cells. Oil Red O staining revealed red color droplets when adipogenic induction happened (20).

In characterization by flow cytometry, cluster of differentiation (CD) surface marker was analyzed by cell harvesting, washing with cytometer buffer [PBS+2% bovine serum albumin (BSA; Biological Industries)] for 5 min at 600 g and incubation with specific labeled antibodies in cytometer buffer at 4°C for 20 min. Primary antibodies with fluorophores were utilized against human cell surface CD antigens CD44, CD73, CD90 and CD105 as mesenchymal stem cell markers, CD34 and CD45 as hematopoietic stem cell markers and Notch1. Data were collected using a FACS Aria II flow cytometer (BD Biosciences, UK) (Table 1) (20).

Table 1: Antibodies used in this study and their sources.

Name	Isotype	Fluorophore	Protein	Source
Anti-CD34	IgG1	PE	Glycoprotein	Bio Legend
Anti-CD44	IgG2b	FITC	Glycoprotein	BD Biosciences
Anti-CD45	IgG2b	FITC	Receptor	Santa Cruz Biotechnology
Anti-CD73	IgG1	PE	5′-Nucleotidase	Bio Legend
Anti-CD90	IgG1	FITC	Glycoprotein	Bio Legend
Anti-CD105	IgG1	APC	Glycoprotein	BD Biosciences
Anti–Notch1	IgG1	APC	Receptor	eBioscience

CD: cluster of differentiation, Ig: Immunoglobulin, PE: Phycoerythrin, FITC: Fluorescein isothiocyanate, APC: allophycocyanin

To determine the growth kinetics of WJSCs, 4×10^4 cells from third passage were seeded in a 24-well culture plate and put in an incubator with 5% CO_2 at 37 °C and saturated humidity. Media change was every 3 days and cell viability and the population doubling time (PDT) were assessed during 7 days applying the GraphPad Prism (GraphPad Software Inc., USA), 0.4% trypan blue solution (Biowest, USA), a phase contrast microscope, and a Neubauer hemocytometer. Each sample was evaluated three times and cells were cryopreserved at a density of 1×10^6 cells/mL in 10% dimethyl sulfoxide (DMSO; MP Bio) (v/v), 50% FBS (v/v) and 40% DMEM (20).

MTT assay was applied to assess the toxicity of recreational dose of 60 µg/mL of methamphetamine (Sigma-Aldrich, USA) as mentioned before (26). The dose of 60 µg/mL of methamphetamine was previously treated with adipose tissue stem cells (AdSCs) (15) and was utilized to expose WJSCs to this dose. Methamphetamine was used while dissolved in normal saline and the defined dose was added twice with a 6 hours interval. The control was cells not treated with methamphetamine and incubated with culture medium for 4 h. For each sample, MTT solution in 0.1 mM PBS was added to each well and then incubated at 37 °C for 4 hours. Cells were enumerated for 8 days, while 3×10^4 cells at 3rd passage/per well were utilized and transferred into 5 wells of 12-well culture plates (One plate was considered control and 4 regarded methamphetamine treated cells). The experiments were repeated three times (15).

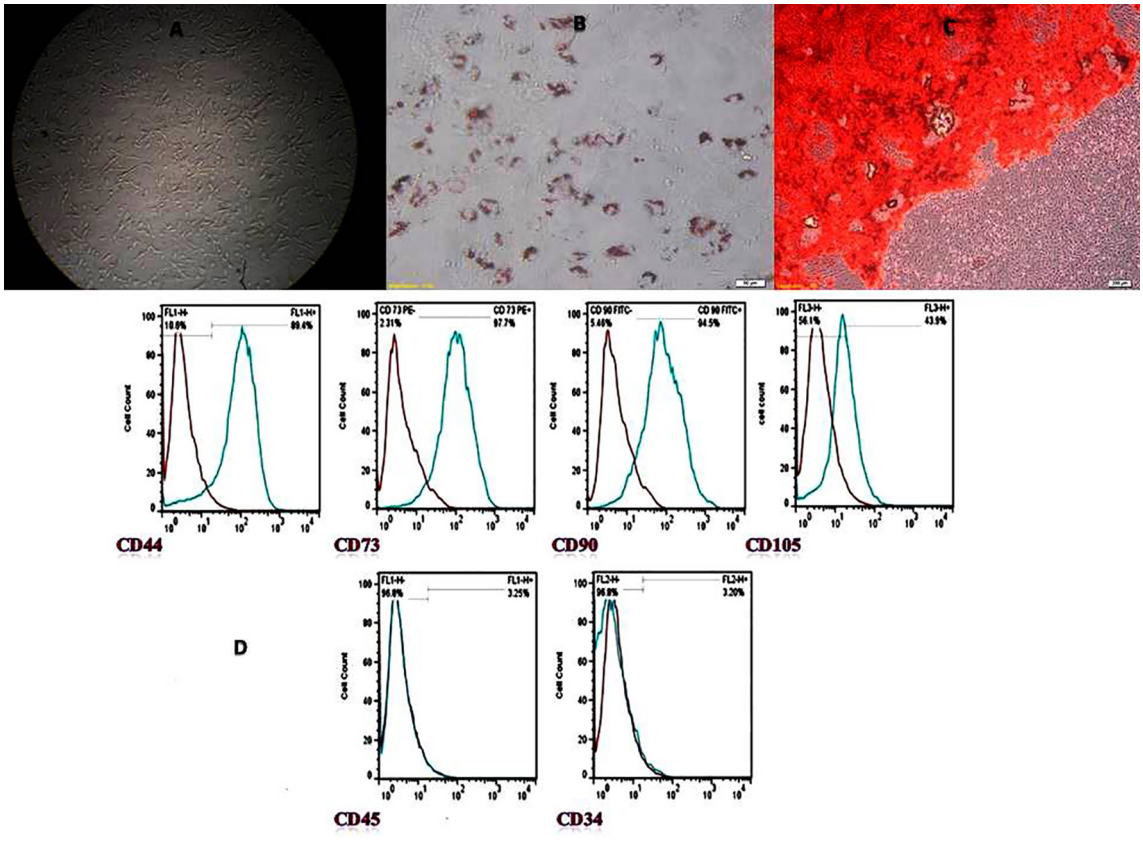

Figure 1: Characterization of Wharton's jelly stem cells (WJSCs): A: Spindle shape in passage 3 (20×), B: Adipogenic induction in red color by Oil Red O staining (40×), C: Osteogenic induction in red color by Alizarin Red staining (40×), D: Positive expression of CD44, CD73, CD90 and CD105 and negative expression of CD34 and CD45 markers.

The normal distribution of data was tested the Kolmogorov-Smirnov test. The data sets were analyzed by one-way analysis of variance (ANOVA). There were two-fold purposes for ANOVA to simultaneously compare two data sets. When a significant difference was detected between treatments, Tukey post-hoc test was applied to find out which treatment differed from the others. A p value<0.05 was considered statistically significant.

Results

The WJSCs were shown to be adherent to the culture plates and were fibroblast-like spindle shape (Figure 1A). A positive adipogenic differentiation was noticed for WJSCs when stained by Oil Red-O illustrating intracellular lipid droplets in red color (Figure 1B). Also, they were positive for osteogenic induction revealing calcium deposits in red color when stained by Alizarin Red after three weeks (Figure 1C). WJSCs displayed positive expression for mesenchymal markers of CD44, CD73, CD90 and CD105 and demonstrated negative expression of hematopoietic markers for CD34 and CD45 (Figure 1D).

WJSCs at passage 3 and culture for seven days denoted to a PDT of 40.1 hours. An increase in cell proliferation was visible till day 6. Later, a decrease in cell proliferation was seen (Figure 2). All experiments for each sample were repeated 3 times.

MTT assay illustrated a reduction in

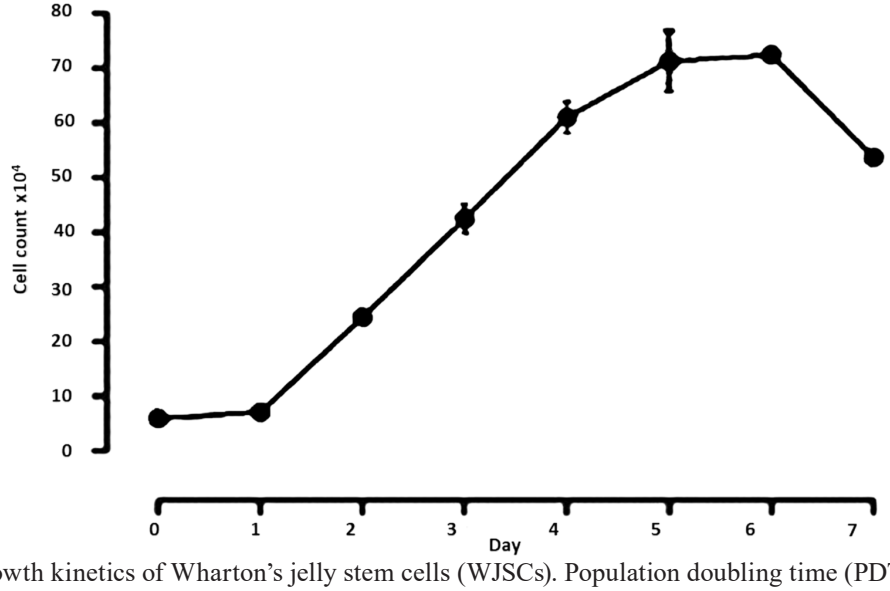

Figure 2: Growth kinetics of Wharton's jelly stem cells (WJSCs). Population doubling time (PDT) was 40.1 h.

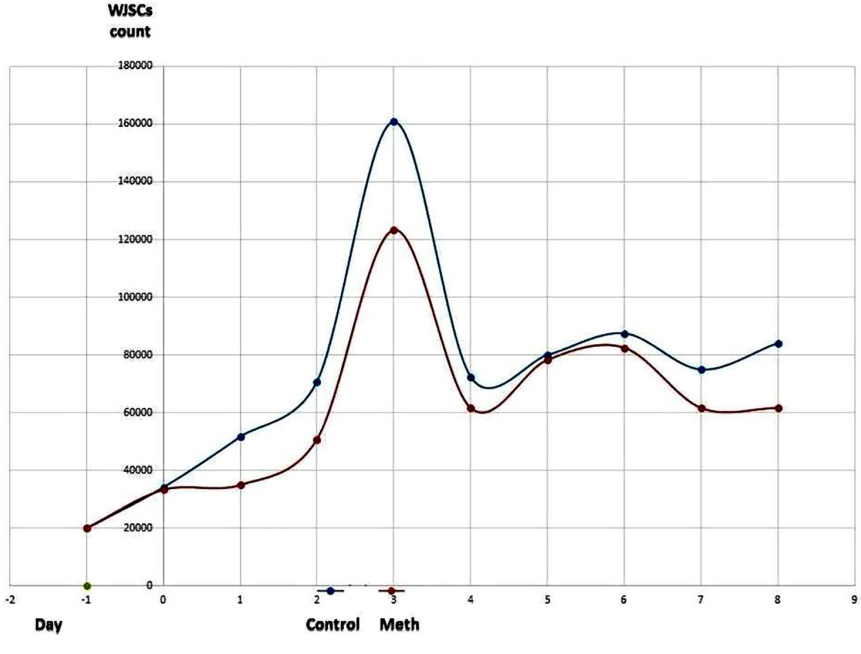

Figure 3: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay comparing the proliferation of Wharton's jelly stem cells (WJSCs) treated with methamphetamine in comparison to untreated cells, while on days 1, 2, 3, 4 and 7; the statistical difference was significant (p=0.01).

proliferation of WJSCs when cells were treated with methamphetamine in comparison to non-treated cells till day 8 (Figure 3). The experiments were repeated 3 times for each sample. On days 1, 2, 3, 4 and 7; they statistical difference was significant (p=0.01) revealing the toxic effect of methamphetamine on proliferation and viability of WJSCs.

Discussion

Drug addiction is still a chronic, debilitating and costly disease impacting many organs (27). Among abused substances, methamphetamine is a psychostimulant drug that is largely consumed for recreational purposes and is of great public health importance, because of high level of methamphetamine-induced dopamine in the brain and the behavioral alteration (28). Methamphetamine or crystal is part of family called non-catecholamines. When it is abused, it can induce euphoria and alertness based on its long half-life action (29). Methamphetamine is mostly metabolized in the liver through: (i) aromatic hydroxylation through cytochrome P450 2D6 to produce primarily 4-hydroxymethamphetamine; (ii) N-demethylation producing amphetamine that is catalyzed by cytochrome P450 2D6; and (iii) β-hydroxylation producing norephedrine (30). About 70% of methamphetamine is excreted in the urine within 24 hours (30-50% as methamphetamine, 15% as 4-hydroxymethamphetamine and 10% as amphetamine) (31). The Final plasma half-life of methamphetamine is about 10 hours and acute effects persist for up to 8 hours (32).

In the present study, we have utilized an *in vitro* model by utilization of WJSCs and have treated the cells with methamphetamine to investigate the impact of this psychostimulant drug on proliferation and viability of the cells. *In vitro* models have already been used in in this relation to assess the side effects and to expand the findings to human organ and tissues (33). Mesenchymal stem cells have been a target of choice for *in vitro* studies and an important tool due to their use in regenerative medicine that can improve tissue repair, regenerate damaged tissues when transplanted, because they have the ability to differentiate into many cell lineages such as bone, cartilage, etc. (34). In this study, we have used WJSCs as a template of mesenchymal stem cells, while these cells are derived from Wharton's jelly that is the mucous ligament in the umbilical cord and contains myofibroblast-like stromal cells, collagen fibers, proteoglycan, and hyaluronic acid that can be surgically employed in regenerative medicine in the treatment of many diseases such as osteoarthritis (35).

Our findings demonstrated the negative impact of recreational dose of methamphetamine on WJSCs that had decreased the proliferation and viability of WJSCs denoting to public health importance of methamphetamine used recreationally. Anari *et al.* when treating adipose tissue stem cells to methamphetamine showed an increase in apoptosis of stem cells and a decline in the proliferation and viability of cells that are in line with our findings (15). Exposure to substances such as cannabis in adipose tissue and bone marrow stem cells has previously described too. Jamshidi *et al.* in two studies reported *in vitro* exposure of adipose tissue stem cells to cannabis with a decrease in cell proliferation, viability and differentiation (13, 14), and similar findings were presented by Sazmand *et al.* when bone marrow stem cells were treated with cannabis (16); and all these data are in line with our findings.

Parsa *et al.* in two studies revealed the negative effect of cannabis extract on SH-SY5Y nerve cells (36, 37). Kamali-Sarvestani *et al.* in two other studies on adipose tissue stem cells exposed to cannabis showed apoptosis and a reduction in cell proliferation (28, 38). Farhadi *et al.* in endometrial stem cells denoted to a decline in cell growth when cells were treated with cannabis (39). All these studies identical to our results confirm the decreasing effect of methamphetamine on cell proliferation and viability.

Conclusion

Our results can be the first study evaluating the effect of methamphetamine on WJSCs at cellular level revealing a decrease in cell proliferation, and viability when cells are exposed and treated with methamphetamine. These findings can be added to the literature especially when methamphetamine is targeted for recreational purposes.

Acknowledgement

We wish to appreciate Shiraz Islamic Azad University for financial support of this study. We are thankful to Shiraz Police Department for dedication of methamphetamine samples.

Conflict of Interest

None declared.

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