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ORIGINAL ARTICLE

In Vitro Assessment of Morphology, Proliferation, Apoptosis and Differential Potential of Dental Pulp Stem Cells, When Marijuana Is Added to Nutrients of Cell Culture Medium

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ABSTRACT

Background: Cannabis, commonly known as marijuana, is widely used for recreational purposes. It has stimulatory effect on appetite, so cannabinoid receptor antagonists have been used to decrease food intake and to act peripherally by rising thermogenesis and energy expenditure to control obesity. This *in vitro* study determined morphological, growth, apoptosis and differential potential of changes in dental pulp stem cells (DPSCs) when marijuana was added to nutrients of cell culture medium.

Methods: Wisdom teeth extracted were used to obtain DPSCs, while characterized morphologically, by osteo- and adipo-inductions and flowcytometry for mesenchymal properties. MTT assay identified optimal concentration of cannabis extract. Cells were treated with 120 and 1000 ng/mL of cannabis during seven days period, while proliferation, apoptosis and differentiation of DPSCs were assessed.

Results: DPSCs were spindle shape and showed mesenchymal characteristics. MTT assay illustrated an increase in cell number until day 5th when DPSCs were treated with 120 and 1000 ng/mL of cannabis, while there was a decreasing trend on day 6th. There was an upregulation of the expression of Bax and COL1A1genes on day 6th when 120 and 1000 ng/mL of cannabis were added to the media in comparison to the control group.

Conclusion: The increase in DPSC proliferation and viability when treated with cannabis denotes to its positive impact on cell proliferation during short term period, while a long term exposure to cannabis resulted in apoptosis and a decrease in cell proliferation. These findings reveal an issue of public health concern and alarm for health authorities.

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Introduction

Cannabis sativa [L.] use, also known as marijuana with increasing decriminalization and legalization is increasing in many countries. Cannabis is a multipurpose crop grown in many countries in Europe, Asia, and North and South America for its flower, fiber, and seed. It has mineral elements of boron (B), sodium (Na), calcium (Ca), magnesium (Mg), potassium (K), zinc (Zn), iron (Fe), and copper (Cu) and 17 amino acids, of threonine, alanine, serine, leucine, isoleucine, lysine, glycine, valine, proline, tyrosine, histidine, arginine, cysteine, methionine, aspartic acid, glutamic acid, and phenylalanine in addition to saturated and unsaturated fatty acids (cis- and trans- isomers) and as fatty acid methyl esters (FAMEs) (1).

It has phytochemicals of delta-9 tetrahydrocannabinol (Δ9-THC), and cannabidiol (CBD). CBD lacks the psychotropic effects of Δ9-THC and with primary non-psychoactive chemicals, while used for recreational, cosmetic, pharmaceutical, and food industries purposes. CBD can also be produced synthetically, yielding a pure form of CBD with pharmacokinetics and effects. Cannabinoids act on two different receptors including CB1 receptors which are located in brain and many peripheral tissues, and CB2 receptors that are primarily found in immune system cells (2) (Figure 1).

In Iran, cannabis is one of the most common illegal drugs abused for recreational purposes and with many adverse effects. Rostam-Abadi *et al.* found the prevalence of cannabis abuse for recreational purposes during the last 12-month to be 1.3% and 0.2% among the male and female Iranian general population, respectively revealing a rise from 0% in 2001 to 0.5% in 2011. From 2016 to 2020, the pooled prevalence estimates of last 12-month cannabis use were 4.9% and 0.3% among males and females of "combined youth groups", respectively. The linear trend of last 12-month cannabis use among males of "combined youth groups" and among female university students showed a significant increasing trend from 2000 to 2020 (3).

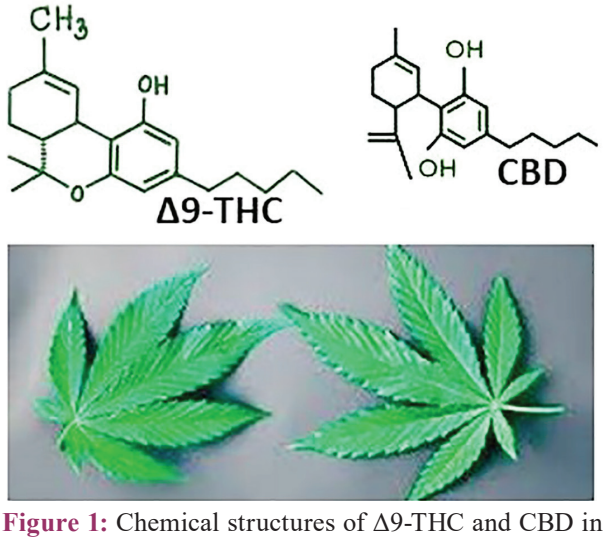

Figure 1: Chemical structures of Δ9-THC and CBD in *Cannabis sativa*.

Cannabis has therapeutic advantages based on its analgesic, antioxidant, anti-apoptotic, anti-inflammatory, neuroprotective, and neuro-modulatory effects (4). It has well-known stimulatory effect on appetite, so cannabinoid receptor antagonists can play a central role by blocking CB1 receptors, thereby decreasing food intake. They may also act peripherally by rising thermogenesis and energy expenditure (5). Cannabis was demonstrated to affect obesity and metabolism when these are modulated by aerobic training (6, 7). Cannabis may have acute and chronic side effects such as anxiety, impaired coordination, suicidal ideations, hyperemesis syndrome, respiratory, cardiovascular and gastrointestinal diseases, neurocognitive impairment and its link with malignancy when used therapeutically (8).

To investigate the impact of cannabis on body cells, mesenchymal stem cells (MSCs) has been utilized *in vitro*. Jamshidi *et al.* used adipose tissue stem cells (AdSCs) (9), Sazmand *et al.* utilized bone marrow stem cells (BMSCs) (10), Farhadi *et al.* employed endometrial stem cells (EndSCs) (11), Parsa *et al.* applied neural stem cells (12), El-Mouelhy et al. used gingival stem cells (13) and Peeri and Koltai studied cancer stem cells (14).

MSCs have been derived from various tissues including adipose tissue (15), bone marrow (16), Wharton's jelly (17), amniotic membrane (18), endometrium (19), menstrual blood (20), periodontal ligaments (21) and dental pulp (22), that possesses anti-inflammatory and immunomodulating properties (23). They are easily cultivated and have been used in clinical trials (24). As there is a need to investigate about adverse effects of cannabis, modeling of *in vitro* studies utilizing MSCs can open a window to study side effects of cannabis. MSCs have been previously mentioned to be a proper *in vitro* model of evaluation for cannabis studies (13).

Despite acknowledgment of the therapeutic and potential adverse effects of cannabis, *in vitro* research about its effect on morphology, proliferation, viability and differential potential of stem cells has not yet matured and there is a need for long-term high-quality study to confidently clarify its effects. So this study was undertaken to determine the impact of marijuana on morphology, proliferation, viability and differential potential of DPSCs when added to nutrients of cell culture medium.

Materials and Methods

Cannabis was provided by permission from Shiraz Police Headquarters for research study purposes. After the preparation of the plant, their authenticity was verified by an expert from herbal center of Shiraz University. They were subjected to a two-week drying process in a dark and dry environment and then changed to powder. A total of 50 grams of the dried powder were further placed in a percolator machine along with 500 mL of 70% ethanol (Merck, Germany) for 72 hours. The ethanol solvent was completely evaporated by rotating the mixture at 50 rpm in a rotary machine (IKA, Germany) at a temperature of 45°C. Finally, the extract provided and dissolved in an ethanol solution to be used later.

Human third molars wisdom teeth were provided from individuals aged between 18 and 30 years and transferred into 15 mL falcon tubes containing Hanks buffered solution to be taken to the stem cell laboratory. A written consent was provided from each individual undergoing tooth extraction. The Ethics Committee of Islamic Azad University approved all experimental protocols. Dental pulp tissues were taken out from extracted teeth using a needle attached to a 1 mL syringe filled with Dulbecco's Modified Eagle's Medium F12 (DMEM-F12, Gibco, Waltham, MA, USA). Under a class II laminar flow hood, the dental pulp was washed three times with PBS and later chopped into small pieces by a sterile blade, filtered and transferred into another 15 mL falcon tube containing 5 mL of DMEM-F12. Then, the falcon content was centrifuged at 200× g for 10 min, the supernatant was removed, and the remained pellet was treated with 1.5 mL of 0.14% collagenase type I (Gibco, Waltham, MA, USA) for 45 min and transferred to a 5% CO_2 incubator at 37°C and saturated humidity. It was centrifuged again at 200× g for 10 min, while the supernatant was taken out, and the remained pellet was suspended in 1 mL of DMEM-F12 culture media containing 10% fetal bovine serum, 1% non-essential amino acids (Sigma, USA), and 1% penicillin streptomycin.

The suspended cells were transferred in a 25 mL culture flask containing 4 mL of DMEM-F12, 10% fetal bovine serum, 1% non-essential amino acids, and 1% penicillin streptomycin. The culture flask was put in a 5% CO_2 incubator at 37°C with saturated humidity, and media change happened every 3 days to reach 80% confluence. Subculturing of cells was performed until third passage at 80% confluence by treating the cells with 0.25% w/v trypsin-EDTA (Gibco, USA). To characterize the cell for mesenchymal properties, DPSCs were evaluated morphologically under an invert microscope (Nikon, Japan) to be spindle shape and pictures were provided by a digital camera (Olympus, Japan).

DPSCs were also characterized by adipogenic differentiation property, while cells were placed in 6-well plates with culture medium. After 80% confluence, media changed was carried out with adipogenic media for 21 days by addition of 15% FBS, 100 nM dexamethasone, 200 µM indomethacin, and 100 µM ascorbic acid (Sigma Aldrich, USA). After 3 weeks, DPSCs were fixed for 20 min in 10% formalin. They were later washed 3 times with deionized water and then were stained for 2 hours with 0.5% Oil Red-O (Sigma-Aldrich, USA). Adipogenic induction appear in red color due to presence of oil droplets in the cells.

DPSCs were characterized by osteogenic induction too by transferring into 6-well plates until 80% confluence. Osteogenic medium contained 15% FBS, 50 µM ascorbic acid (Merck, Germany), 100 nM dexamethasone (Sigma Aldrich, USA), and 10 mM glycerol 3-phosohate (Merck, Germany) that were added to culture media for 3 weeks. Media alteration was every 3 days and after 21 days, DPSCs were fixed in 10% formalin for 20 min, washed three times with deionized water, and finally stained with Alizarin Red solution (Sigma-Aldrich, USA). Osteogenic induction appear in red color based on calcium deposit and calcification in differentiated cells.

DPSC were also characterized by flowcytometry. After 21 days, trypsinized cells were suspended in PBS at 1×10^6 cells/mL. Aliquots of 100 µL of cell suspension was provided in each Falcon polystyrene fluorescent activated cell sorter tube and incubated for 30 min in dark at 4°C with fluorescein-conjugated antibody for CD73 and CD90 as mesenchymal markers and CD34 and CD45 as hematopoietic markers. Then, DPSCs were washed with PBS, and centrifuged identically. The supernatant was removed and the remained pellet was suspended in an aliquot of 100 µL PBS and was run applying a flow cytometer (Becton-Dickinson, USA) (Table 1).

Trypan blue exclusion test using 0.4% trypan blue in PBS was undertaken to assess the number of viable cells. To assess the cell growth, MTT assay (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, Sigma-Aldrich, USA), was undertaken. Cells were seeded into 96-well plates at a density of 5000 cells per well in 200 µL of culture medium, while various concentrations of 20, 100,110,120, 150, 1000 ng/mL of cannabis was added to the media. After 24, 48, and 72 hours, 20 microliters of MTT were added for each 200 µL of the culture medium in each well. Following four hours incubation at 37°C with 5% CO_2 (Memmert CO_2 incubator, Germany), the supernatant was discarded, and the

Table 1: Antibodies used and their sources.

Name	Isotype	Fluorophore	Protein	Source
Anti-CD34	IgG1	PE	Glycoprotein	Bio Legend
Anti-CD45	IgG2b	FITC	Receptor	Santa Cruz Biotechnology
Anti-CD73	IgG1	PE	5′-Nucleotidase	Bio Legend
Anti-CD90	IgG1	FITC	Glycoprotein	Bio Legend

CD: Cluster of differentiation, FITC: Fluorescein isothiocyanate, IgG: Immunoglobulin G, PE: Phycoerythrin.

Table 2: Primers used in the present study.

Gene	Primer sequence
Bax	Forward: 5'-GCCCTTTTGCTTCAGGGTTTCA-3'
	Reverse: 5'-CAGCTTCTTGGTGGACGCAT-3'
COL1A1	Forward: 5′-GACGAAGACATCCCACCAAT-3′
	Reverse: 5'-TCGGTGGGTGACTCTGAG-3'
GAPDH	Forward: 5'-GGCTGTTGTCATACTTCTCATG-3'
	Reverse: 5'-CCATCTTCCAGGAGCGAGA-3'

COL1A1: Collagen, type I, alpha 1, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

MTT formazan compound dissolved in 200 µL of dimethyl sulfoxide for 1 hour at room temperature was trapped within the cells. Subsequently, the optical densities were measured at 540 nm using a multiwell spectrophotometer (Bio Rad, USA) with a reference wavelength of 650 nm.

For gene expression analysis, the total cell RNAs were isolated by RNA extraction kit (Cinnagen Inc., Iran), while RNA integrity, purity, and concentration were examined at optical density of 260/280 applying 1% agarose gel electrophoresis. According to manufacturer's guideline, complementary DNA (cDNA) was provided employing RevertAid™ First Strand cDNA Synthesis kit (Fermentas Inc.) by 1 µg RNA. According to guideline of RealQ Plus 2x Master Mix Green (Ampliqon Inc.), quantitative real-time PCR (qPCR, Biosystems StepOne™ Instrument, ABI, Step One, USA) was utilized for gene expression analysis of the primer pairs for GAPDH, Bax, and collagen type I alpha 1 (COL1A1) that was displayed in Table 2. The GAPDH was considered the housekeeping gene as the internal control. qPCR was set at 94°C for 10 minutes, then at 94°C for 40 cycles of 15 seconds, at 60°C for 60 seconds and extension steps. All reactions were done in triplicate. Following each run, gel electrophoresis and melting curve analysis were applied to verify specific amplification of targets. The amplification signals of different samples were normalized to GAPDH Ct (cycle threshold), and then delta-delta CT (2 ΔΔCt) method to compare the mRNA level of test versus control represented as fold change in data analysis.

All data were presented as mean±SEM of three independent experiments. Findings were analyzed by the one-way analysis of variance (ANOVA) using Prism version 6.0 software (GraphPad Software Inc., San Diego, CA, USA). The *p* value was considered statistically significant when it was ≤0.05.

Results

Figure 2 displays DPSCs to be spindle shape and fibroblast like, while they were adherent to the culture flasks in all passages, which is a distinctive feature of mesenchymal characteristics of the cells (Figure 2A). The cells demonstrated a positive differentiation characteristic for osteogenic induction indicating presence of calcium deposits in red color in DPSCs after staining with Alizarin Red (Figure 2B). DPSCs illustrated positive adipogenic induction after staining with Oil Red-O revealing presence of intracellular lipid droplets in red color (Figure 2C). A positive expression of CD73 and CD90 as mesenchymal markers was shown, while DPSCs were negative for hematopoietic markers of CD34 and CD45 (Figure 2D).

The growth curve provided by plotting the cell count over a period of seven days was presented in Figure 3, while the horizontal axis represents time in days, and the vertical axis denotes to the cell count. MTT assay illustrated an increase in cell number on days 3rd, 4th and 5th when DPSCs were treated with 1000 ng/mL of cannabis, while there was a decreasing trend after day 6th. This increase for 120 ng/mL of cannabis was seen on days 4th and 5th, but there was a decreasing trend after day 6th for both doses of cannabis (120 and 1000 ng/mL) (p=0.01) revealing the toxic effect of cannabis on proliferation of DPSCs.

There was an upregulated expression of Bax gene on 6th day when 120 ng/mL of cannabis was added to the media in comparison to the control group, but the difference was not statistically significant (p=0.07). This upregulation for 1000 ng/mL of cannabis was

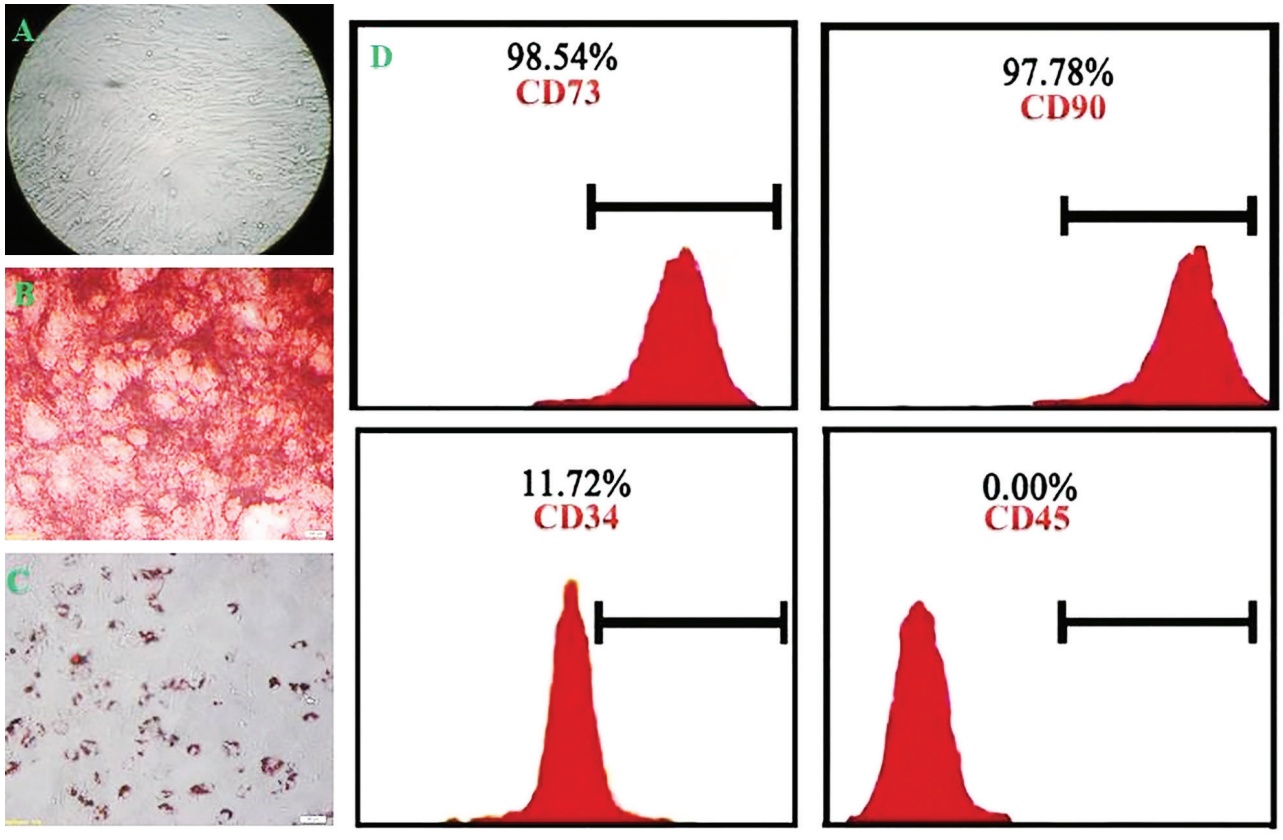

Figure 2: Cell characterization: A: Spindle shape in passage 3 (20×), **B:** Positive osteogenic differentiation in red color by Alizarin Red staining (40×), **C:** Positive adipogenic induction in red color by Oil Red O staining (40×), D: Positive expression of CD73 and CD90 as mesenchymal markers and negative expression of CD34 and CD45 as hematopoietic markers.

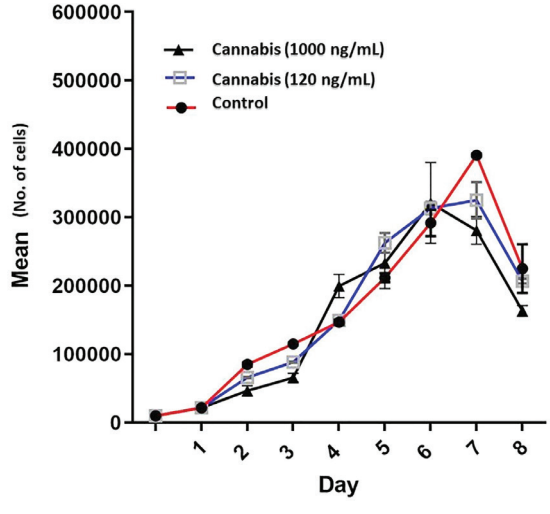

Figure 3: Growth curve of dental pulp stem cells treated with methamphetamine. The control group, the group treated with a concentration of 120 ng/mL of cannabis, and the group treated with a concentration of 1000 ng/mL of cannabis.

statistically significant in comparison to the control group (p=0.0001, Figure 4). Expression of COL1A1 gene increased when 120 ng/mL of cannabis was added to the media, but no statistically significant difference was visible in comparison to the control group (p=0.30). However, when 1000 ng/mL of cannabis was added to the media, a statistically significant increase in expression of COL1A1 gene was observed, and this difference was statistically significant when compared to the control group (p=0.01, Figure 4).

Discussion

MSCs as multipotent stromal cells have captured attention in the scientific world based on their differentiation potential into osteoblasts, chondroblasts and adipocytes and possible transdifferentiation into other cells such as neurons, hepatocytes, cardiomyocytes and endothelial cells. Several growth and angiogenic factors, and cytokines have been discovered in MSC secretome (25, 26). These adult stem cells are a resource for living organisms allowing the repair and/or regeneration of an injured tissue (27, 28). Among MSCs, DPSCs are unique as they are originated from the ectomesodermal embryonic tissue that forms the neural crest and they can also give rise to odontoblasts and nerve cells of ligodendrocytes, glia cells and astrocytes (29-31). These cells have been employed *in vitro* as a model for pharmacological and therapeutic approaches including *Cannabis sativa* (32). El-Mouelhy *et al.* evaluated the *in vitro*

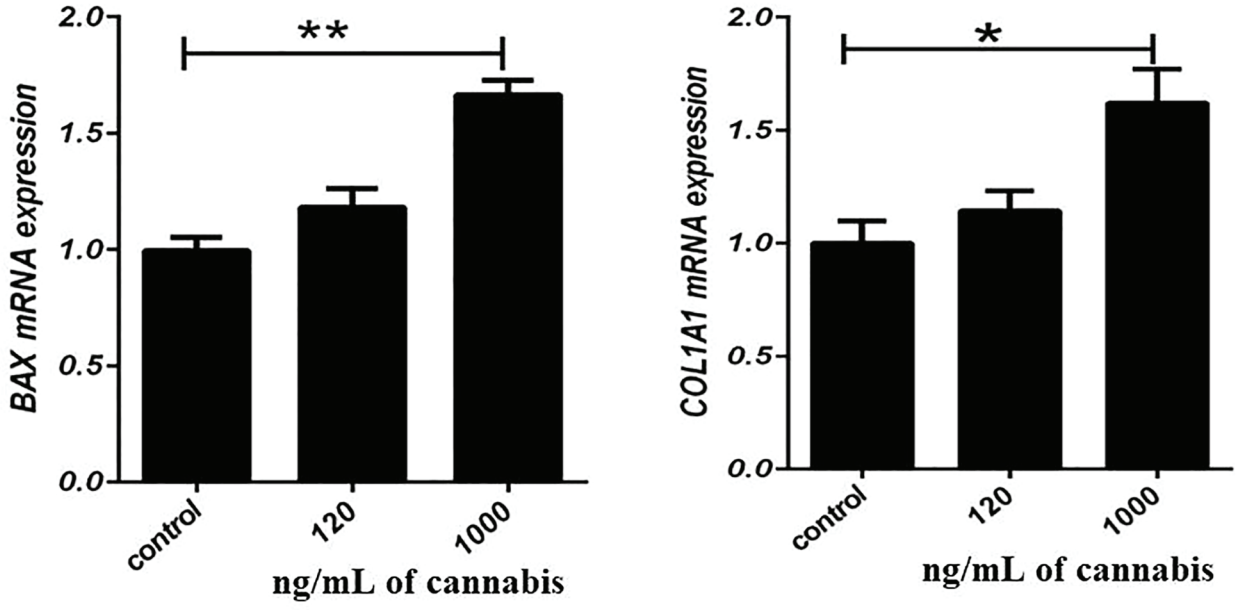

Figure 4: Bax and COL1A1 gene expression when cannabis was added to the media.

effect of cannabis on gingival MSCs and showed DNA damage and cellular dedifferentiation with negative impact on cellular proliferation and viability of MSCs (13).

The results of our study denoted to mesenchymal properties of DPSCs that has been described before morphologically, by osteo- and adipo-genic properties and via flowcytometry or PCR (33- 35). Our findings revealed that cannabis at doses of 120 and 1000 ng/mL when added to culture media increased cell proliferation in a dose dependent manner until day 5th, while there was a decreasing trend on day 6th for cell proliferation and viability revealing the short term positive impact of cannabis on cell proliferation and negative effect in long term period. Farhadi *et al.* reported an increased proliferation rate of EndSCs treated with cannabis until day 4th (11). Parsa *et al.* used SH-SY5Y cells treated with cannabis and illustrated an increased proliferation rate until day 5th and a decrease on the 6th day (12). Jamshidi *et al.* employed AdSCs exposed to cannabis and found an increase in proliferation of AdSCs until 5th day and a decrease on day 6th in absence of any negative impact on cell differentiation (9, 36). Sazmand *et al.* found an increase in proliferation of BMSCs when cannabis was added to the media until 5th day and a decreased proliferation rate on day 6th (10). El-Mouelhy *et al.* used gingival MSCs for the effect of cannabis addition to the media and showed DNA damage and cellular dedifferentiation with negative impact on cellular proliferation and viability (13). These finding on AdSCs, BMSCs, EndSCs, SH-SY5Y cells and gingival MSCs are in agreement with our results that the initial effect of cannabis on proliferation of DPSCs was positive and dose dependent, while long term impact might decrease cell proliferation and viability due to an increased apoptosis in DPSCs.

Regarding apoptosis, many researchers have studied the in vitro effect of abused substances such as methamphetamine for apoptosis on different stem cells (37-39). For cannabis impact, Kamali-Sarvestani *et al.* showed an upregulation of the expression of Bax and COL1A1genes of AdSCs on day 6th when cannabis was added to the media in comparison to the control group (40). El-Mouelhy *et al.* used gingival MSCs for the effect of cannabis addition to the media and showed DNA damage (13). Identically, we showed an increase when cannabis was added to the media in the expression of Bax and COL1A1genes of DPSCs when compared to the control group on day 6th.

Conclusion

The increase in DPSC proliferation and viability when treated with cannabis denotes to its positive impact on cell proliferation during short term period, while a long term exposure to cannabis resulted in apoptosis and a decrease in cell proliferation. These findings reveal an issue of public health concern and alarm for health authorities.

Acknowledgement

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Authors' Contribution

All authors have read and approved the manuscript. DM, SZ and FKB designed the study. MA and SZ were responsible for cannabis preparation. MA, DM, SZ, MH, SSM, GM and ASM did dental pulp extractions, culture, differentiation and MTT assays for cannabis exposure. MI and IJ conducted real time PCR and gel electrophoresis. IJ, SSH carried out the data analysis. DM and FKB wrote the first draft of the manuscript and all named authors contributed in revising and finalizing the manuscript.

Conflict of Interest

The authors state that they do not have any conflicts of interest. Davood Mehrabani, as the Associate Editor, was not involved in any stage of handling this manuscript. A team of independent experts were formed by the Editorial Board to review the editor's article without his knowledge.

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