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Effect of hydroalcoholic extract of Cannabis (Cannabis sativa L.) on morphology and the process of human adipose-drived mesenchymal stem cell growth

Mehregan Jamshidi[1,2], Seyed Ebrahim Hosseini[2], Davood Mehrabani[3], Masoud Amini[4]

ABSTRACT

Background: Today, cannabis sativa L. is the most consuming drug trafficking plant in the world after nicotine and alcohol.

Aims: Given the properties of cannabis and psychoactive compounds of this opiate drug, including hashish, as well as the importance of human adipose-derived mesenchymal stem cells in the restoration and regeneration of organs and its application in tissue transplantation and tissue engineering, the current paper investigates the effect of hashish (hydroalcoholic extract of cannabis) on the morphology and growth process of human adiposed-drived mesenchymal stem cells in vitro.

Methods: Human adipose-drived mesenchymal stem cells were extracted from the abdominal fat by the enzyme digestion of the patient's abdominal region. MTT test was performed to determine the best concentration of cannabis extract. Cells were treated for seven days at 100 ng / ml and 1000 ng / ml, and propagation, growth and viability of the cells were evaluated.

Results: The results show that cannabis extract reduces the proliferation of human adipose-drived mesenchymal stem cells as time-dependent. Also, the effect of cannabis extract on mesenchymal cells depends on concentrations. High concentrations have a greater negative effect on the growth of the cells. The results of this study showed that the morphology of human adipose-drived mesenchymal stem cells did not change after exposure to cannabis extract.

Conclusion: Although in the first days, the growth process is elevated and the cells are in the euphoric phase, apoptosis increases with time as consumption increases due to toxicity. The effects of cannabis on fat cells indicate a decrease in the volume of adipose tissue and, consequently, in the consumer's slimming.

Keywords: Cannabis, mesenchymal adipose-drived cells, growth process, morphology, mitosis

BACKGROUND

The cannabis belongs to the Canabinous family with a species called Cannabis sativa L. The female flower and the dried leaves of this plant are called marijuana and it has many cannabinoid compounds (1-2). Hashish is a resin secretion of this plant, produced in its trichomes, and has a high content of delta-9-tetrahydrocannabinol (THC) (3-6).

Mesenchymal stem cells are one of the most important adult stem cells that have multivariate characteristics, fibroblastic morphology, high proliferation potential, and adhesion properties to the floor of plastic containers, self-renewal, and the ability to differentiate into multiple cell lines (6-10). Adiposed-drived mesenchymal stem cells, in addition to the mentioned characteristics, also have other advantages, such as the lack of ability to proliferate and differentiate in long time cultures and the availability of large reserves and easy access to a large amount of fat through sampling and liposuction (11-15). Because of these properties are very useful in therapeutic and therapeutic medicine (16-19).

[1] *Department of Biology, Fars Science and Research Branch, Islamic Azad University, Shiraz, Iran*
[2] *Department of Biology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.*
[3] *Burn and Wound Healing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.*
[4] *Laparascopy Research Center, Department of Surgery Shiraz University of Medical Sciences, Shiraz, Iran*

Correspondence: *Seyed Ebrahim Hosseini*
Department of Biology, Shiraz Branch, Islamic Azad University, Shiraz, Iran

E-mail: ebrahim.hossini@yahoo.com

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The precise mechanisms through which cannabinoids mediate immunosuppression are mainly divided into four categories: apoptosis, prevention of proliferation, suppression of cytokines and chemokines production, and suppression of T regulator cells induction. THC inhibits the proliferation of lymphocytes in the culture medium (20). It also induces apoptosis and inhibits the survival of cells and invasive effects of tumor types (21). Also, invitro rate of proliferation of commercially derived mesenchymal cells derived from adipose and bone marrow was significantly higher than that of primary culture cells (22). Cannabinoids can induce apoptosis, inhibit proliferation, angiogenesis, and metastasis in glioma cells, and have less toxic effects than those used in chemotherapy. These materials have anti-proliferation and anti-angiogenesis properties in the culture medium and live cancer induction models. Cannabinoids regulate cellular messenger pathways that relate to viability, invasion, angiogenesis, metastasis, etc. (23). Dependent to time and dose the cannabinoid CBD inhibits proliferation of neuroblastoma cells and induce apoptosis in them (24). Given the properties expressed for the cannabis plant and the compounds of this opiate drug, including hashish, as well as the importance of human adiposed-derived mesenchymal stem cells in the restoration and regeneration of organs and its application in tissue transplantation and tissue engineering, the current paper investigates the effect of cannabis on the growth and viability rate of human adiposed-derived mesenchymal stem cells.

METHOD

To prepare a psychoactive cannabis plant, the correspondence with the Counter Narcotics Headquarters was conducted through the Islamic Azad University of Shiraz. After the preparation of the plant, the nature of the cannabis was confirmed by the expert of the biosystematic center of Shiraz University of Medical Sciences.

The leaves were placed in a dark and dry place for two weeks to dry completely. First, 50 g of dried powder was placed in a percolator machine for 72 hours in 500 cc of ethanol 70% (Merck-Germany). After this time, the solvent was rotated 50 rpm in a rotary machine (IKA-Germany) at 45 °C to completely evaporate the solvent (ethanol). Finally, the doses of this extract were dissolved in ethanol and distilled water.

Adipose tissue samples were taken from the abdominal region of a 40-year-old man with complete satisfaction, who underwent abdominoplasty. The specimen was put in sterilized Falcon (BD-USA) containing phosphate buffer saline and one percent penicillin streptomycin antibiotic and antifungal amphotericin and quickly transferred to the cell culture laboratory of the Stem Cell Technology Research Center of Shiraz University of Medical Science.

In order to remove erythrocytes and leukocytes, the fat sample was washed several times with PBS (Gibco-USA) and the supernatant was discarded. About 5 to 6 ml collagenase enzyme 0.2% type 1 was added and stored at water bath at 37 °C for 30 to 60 minutes. Falcon was stirred every 5 minutes to increase the rate of fat tissue decomposition. Three times the volume of enzyme, culture medium was added to neutralize the enzyme. After passing through the 0.7 µm filter, the cells were separated from the debris. The resulting cell soup was centrifuged at room temperature at 1200 rpm for five minutes. This procedure was repeated 2 to 3 times. Oil and supernatant adipose tissue was gently removed. Falcon deposition containing depleted fat cells was retained. The cells were then transferred to a 75 flask (Orang-USA) for cell culture containing DMEM and 10% FBS (Biowest-USA). Finally, the flask containing the cells was placed in a 37°C incubator containing 5% CO2 for 24 hours. The medium was then replaced every three days, and after reaching 80 to 90% of cell confluencey, the cells had been passaged in order to be used in passage 4.

The MTT assay was used to evaluate the bioavailability and proliferation of mesenchymal stem cells and to determine the probable toxicity. At first, 10000 cells were placed in each well of 96-well plate (Invitrogen-USA). After 24 hours the previous culture medium was replaced with new medium with concentrations of 20, 30, ..., 150, 200, 300, ... , 600, 1000, 2000, ..., 6000, ng/ml and incubated for one day at 37°C and 5% CO2 (CO2 Incubator-MEMMERT GERMANY). For each 200µl of the culture medium, 20 microliter of MTT was added to each well of the plate and incubated for 4 hours; then was centrifuged for 5 minutes. The supernatant medium was removed and 200µl DMSO (Sigma-Aldrich-Germany) was added to each well. Optical absorption at 570nm was read by the Elisa plate reader (Polarstar Omega-BMG LABTECH-Germany).

The viability of the cells was investigated using three groups based on dose and time. These patterns consisted of an exposure and counts after 24 hours, an exposure and counts after 72 hours, and exposure per day for 3 days and counting after 72 hours. To compare the effects of different dose ranges, doses were grouped to 30-150, 150-1000, and 1000-6000. Accordingly, the appropriate concentrations were selected from the range of 30-150 ng/ml in this study. The growth curve was examined for all doses, in the first three days. There were no significant differences. Since using all of doses was repetitive and time consuming, just doses of 100 ng/ml and 1000 ng/ml were used. The dose of 100 ng/ml was chosen as a dose in a suitable and desirable range and a dose of 1000 ng/ml was out of the optimal range.

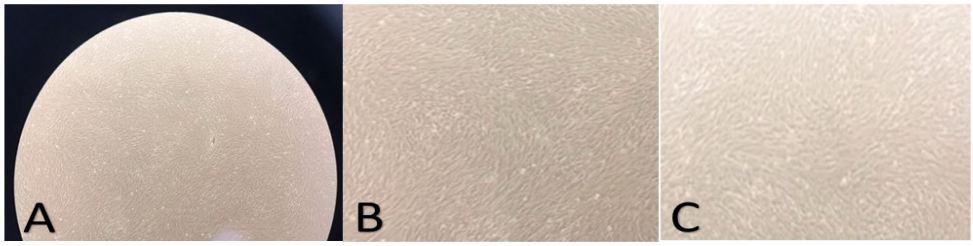

Figure 1: *Cells in the 4th passage with a high density of 4x magnification, a) control group, b) treated group with a concentration of 100ng/ml, c) treated group with a concentration of 1000ng/ml*

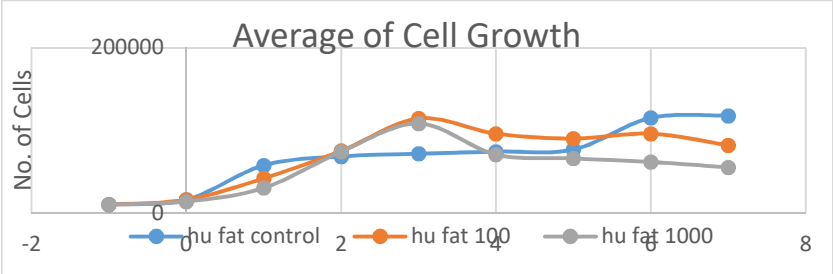

Figure 2: *Average of cell growth curve of control group, treated group with a concentration of 100ng/ml, treated group with a concentration of 1000ng/ml*

In order to evaluate the growth process, three 24-well plates containing adipose-drived mesenchymal stem cells were used. One plate for control group, one for 100 ng/ml dose, and another one for 1000 ng/ml dose of cannabis in the fourth passage were prepared. Each well contained 10000 cells per ml of complete culture medium. All plates were placed in an incubator. All treatment groups were exposed each day. From the next day, three wells from each plate were considered, their culture medium was removed, cells were washed with PBS, and separated from the culture flask with Trypsin/EDTA. After centrifuge, the supernatant was removed and a specific volume of complete culture medium was added to the resulting cell precipitate. Cells were completely suspended by pipetting. Cell counting and PDT measurement were performed. Cells were counted under the microscope every day after staining with Trypan Blue (Merck-Germany), by neobar lam (HBG-Germany). After calculating the number of cells of each well, the average number of cells was taken as the number of cells in every day. In other wells, the medium was changed as well. Cell counting was continued constantly for seven days. By the end of the seventh day and after the last counting, the normal growth curve was drawn according to the data obtained from the count of the control and treatment cell plates.

The size and the shape of the cells in the culture medium was examined every day before cell counting by the Invert microscope (Nikon ECL IPSE TS100-GAPAN).

Statistical analysis was performed using SPSS-23 for parameters such as percentage of viability and growth trend. One-way ANOVA test was used for statistical analysis of the percentage of viability. Growth process was analyzed using one-way ANOVA test and Tukey's post hoc test.

RESULTS

In the results of One Way Anova test, in all groups, the significant level of F (Fisher test) was less than 0.05 (P = 0.001) based on the percentage of viability of the cells. Therefore, the difference between the groups average was confirmed. Treated cells in a suitable culture medium with cannabis extract had a -fibroblastic morphology with no change in size and appearance of mesenchymal stem cells comparing to the control group (**Figure 1**). The growth curve was plotted based on the average number of cells counted in each group in seven days. In this diagram, the horizontal axis represents the time in a unit of day and the vertical axis represents the number of cells (**Figure 2**).

The time required for population doubling time over seven days in the three groups (control group, treated with 100ng/ml and 1000ng/ml cannabis extract) was obtained, as shown in **Table 1**. One-way ANOVA results show that the F-test (Fisher test) is not less than 0.05 (P=0.360) for all groups. Therefore, the absence of a difference between the mean growths of groups is confirmed. The mean values of growth in each groups are different (**Figure 3**). One-way ANOVA test results for growth trend after 3 days of exposure in all groups have a significant level of F-test (Fisher test) and is

Table 1: *PDT values for the three groups: control group, treated with 100ng/ml, and treated with 1000ng/ml*

Days	control	100ng/ml	1000ng/ml
0	0	0	0
1	9.51	11.65	15.06
2	17.31	16.51	16.62
3	25.33	20.49	20.96
4	33.2	29.44	34.06
5	40.83	37.85	44.13
6	40.86	44.16	54.86
7	47.25	55.44	68.29

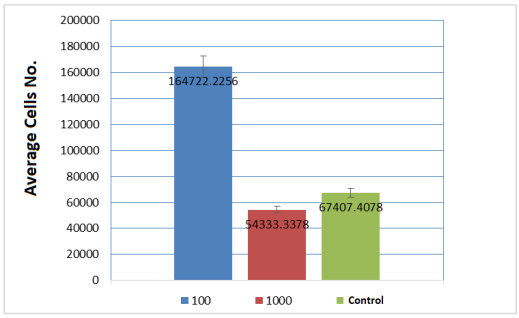

Figure 3: *Mean growth in different groups*

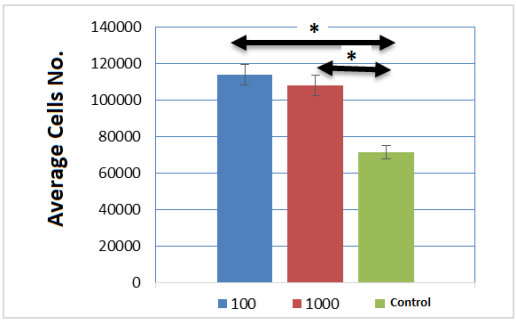

Figure 4: *Mean growth trend among groups after 3 days of exposure*

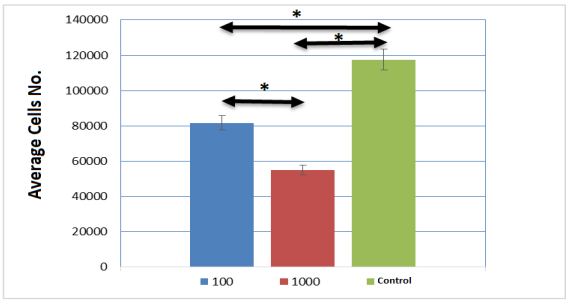

Figure 5: *Mean growth trend among groups after 7 days of exposure*

less than 0.05 (P = 0.001). Thus, the difference between the groups' mean is confirmed. According to Tukey's post hoc test, treatment groups are located in one class. **Figure 4** shows the mean growth diagram in different groups after 3 days of exposure. One-way ANOVA test results for growth trend after 7 days of exposure in all groups had a significant level of F-test (Fisher test) less than 0.05 (P=0.001). So the difference between the mean of the groups is confirmed. According to Tukey's post hoc test, all groups are placed in distinct classes. **Figure 5** shows the mean growth diagram in different groups after 7 days of exposure.

DISCUSSION

The current paper investigated the effect of hydroalcoholic extract of cannabis on the growth of human adipose-drived mesenchymal stem cells. MTT test was used to study the effect of cannabis extract on cell proliferation and growth

process. According to the results of the test of the toxicity of cannabis extract, 100 ng/ml was a euphoria dose and 1000ng/ml was toxic and out of optimal range. The results of the morphological study of cells showed that exposure to different doses of cannabis extract did not affect the shape and size of human adipose-drived mesenchymal stem cells. After exposure, the mesenchymal cells also retained their spindle-shape and semi-fibroblastic appearance and adhered to the plastic medium container floor.

The cell number at different days of exposure and the population doubling time of the cells in treatment groups were compared with the control group in order to investigate the growth trend and proliferation of stem cells. The results of the growth curve showed that all groups entered the lake phase at the beginning of the exposure, so that they could adapt themselves to the environment in order to grow. In the treatment of cannabis extract at doses of 100 and 1000 first increase in the number of cells was observed. As a result of treating cells with 100ng/ml and 1000ng/ml of cannabis extract, due to euphoric effect of the drug at first an increase in the number of cells was observed. But after that due to the toxicity effect of drug because of apoptosis increase, the number of cells decreased.

From the first to the third day, cells in both groups treated with doses of 100ng/ml and 1000ng/ml showed an increasing trend and entered a progressive phase. The maximum number of cells was observed in third day. After the third day, the number of cells in the treatment groups showed a decreasing trend, with a faster speed in dose of 1000ng/ml comparing to that of 100ng/ml.

On the first day of exposure, the cell population in the treatment groups was less than the control group and the population doubling time was more. The growth trend was slow but incremental and the growth curve in the treatment groups showed a slope less than that of the control, which is due to the efforts of the cells to adapt to the culture medium containing cannabis extract. The statistical results of one-way Anova test showed no significant difference in mean growth in the first day between groups.

On the second day, growth curve in both treatment groups showed an increasing trend compared to the control group and the population doubling time was less, which indicates the effect of cell growth enhancement by cannabis extract.

On this day, the slope of growth curve in treatment group with 100ng/ml was higher than the dose of 1000ng/ml, indicating that the dose of 100ng/ml of cannabis extract is the effective dose which causes euphoria phase and dose of 1000ng/ml is the toxic dose which shows it's toxic effect by the end of the third day of exposure.

In the cell growth curve, the maximum growth of cells in both treatment groups is observed on the third day.

The statistical results of Anova one-way test showed a significant difference in the mean cell growth in the third day between the treatment groups and the control group. But there is no significant difference between treatment groups.

On the 4th to 7th day, the growth curve of the cells in the treatment groups showed a decreasing growth trend, indicating a significant decrease in the cell population in exposure to cannabis extract in these days. There is a significant decrease in the growth rate of the cells in the treatment group with a dose of 1000ng/ml of cannabis extracts comparing to the treatment group with a dose of 100ng/ml, indicating a greater toxicity effect of 1000ng/ml than 100ng/ml. 100ng/ml induces euphoria phase in cells, which time-dependently shows its toxic effects in a slower slope. However, 1000ng/ml is time-dependently the toxic dose. This indicates an increase in the toxicity of cannabis extract and apoptosis in cells over time.

The population doubling time on days 4th and 5th was lower in the group exposed to 100ng/ml of cannabis extract than the control group, but in the group exposed to the 1000ng/ml, this time increased comparing to the control group. On the sixth and seventh days, the population doubling time increased in two treatment groups comparing to the control group.

Comparing the two treatment groups, it was observed that the cell population in the 1000ng/ml was always less than the 100ng/ml and the population doubling time was more, which confirmed the toxicity of the 1000ng/ml comparing to 100ng/ml at the same time.

The results of the growth curve indicate that the maximum cell growth is seen in the treated cells with cannabis extract on the third day of exposure, while in the control group, this maximum is seen on the 6^{th} day. Based on these observations, it can be concluded that the consumption of cannabis extract decreases the time to reach the maximum cell growth.

The statistical results of Anova one-way test showed a significant difference in mean growth on the 7^{th} day between different groups. Thus, there was a significant difference between the control group and the treatment groups as well as between the two treatment groups.

CONCLUSION

The current paper shows that the morphology of human adipose-derived mesenchymal stem cells didn't change after exposure to cannabis extract. Although the growth trend is increasing in the first days, apoptosis increases by increasing the dose amounts. The adverse effects of cannabis on the fat cells indicate a decrease in the volume of adipose tissue and consequently in the consumer's slimming. Cannabis extract had a negative effect on the proliferation of adipose-derived mesenchymal stem cells due to increasing apoptosis.

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PEER REVIEW

Not commissioned

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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ETHICS COMMITTEE APPROVAL

This research has been approved by the Committee on Ethics of Islamic Azad University of Shiraz.

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