Cellular and Molecular Biology

Khaleghi *et al. Cell. Mol. Biol.*2016, 62 (11): 76-80
ISSN: 1165-158X
doi: 10.14715/cmb/ 2016.62.11.13

Original Research

Induction of apoptosis by opium in some tumor cell lines

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Abstract: The current study is aimed at investigation of the opium effects on the apoptosis of different cell lines in culture medium and compares such effects with one another. The study is carried out on over 8 cell lines (AA8, AGS, Hela, HepG2, MCF7, N2a, PC12, WEHI). A 2.86 x 10^{-4} g/ml opium concentration was prepared and added to the culture medium of the cell lines for 48 hours. Cytotoxicity was tested by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The apoptotic effect of opium on the cell lines was analyzed by Annexin-PI test. Opium with concentration of 2.86 x 10^{-4} g/ml in 48 hours significantly induces apoptosis in certain cell lines (i.e. AA8, N2a, WEHI), apoptosis and necrosis in some others (i.e. Hela, HepG2, MCF7, and PC12), and also solely necrosis in the AGS cell line. One could infer that the usage of opium with different levels in different tissues leads to certain disorders in some tissues and may have therapeutic effects under distinctive conditions (i.e. unchecked growth of cells) as confirmed by the results.

Key words: Opium, Apoptosis, Necrosis, Cell lines, Flow-cytometry.

Introduction

The mechanism of apoptosis or programmed cell death is one of the primary ways of removing unwanted cells in the body of multicellular organisms (1-3). The disruption of the rate of this phenomenon, with either incremental or reductive trend, leads to cancer and diseases such as Alzheimer and Parkinson (4, 5). The identification of this cellular process and the ways of controlling it will pave the way for development of anti-cancer and anti-inflammatory drugs (6). Different studies have shown that any factor inside or outside the cells inhibiting the natural growth and evolution of the cells might provide proper conditions for their apoptosis (7). The apoptotic effects of alkaloids such as morphine, heroin, codeine, papaverine and noscapine has been proved (8) the production of which is done through opium as the raw ingredient. Opium contains morphine (8-17%), noscapine (1-10%), papaverine (0.5-1.5%) and codeine (0.7-5%), also it has highly varied pharmacological and pathological effects (9). Our previous study showed that opium can induce apoptosis in brain and liver tissues of rats and Jurkat cells (10, 11). The previous studies on different alkaloids of opium such as morphine, noscapine, papaverine, and codeine have shown initial increase and consequent resumption of apoptosis in different cells. Among many studies representing the apoptotic effects of morphine on different cells such as neurons (12), the general conclusion inferred is that this compound causes an increase in the synthesis of pro-apoptotic regulatory proteins such as P53 and Bax in exposed cells (13). Another study reported that morphine leads to the induction of apoptosis in diverse levels in HL-60 and A549 cell lines and necrosis in MCF7 cell line (14). The apoptotic roles of some other primary derivatives of opium such as noscapine and papaverine have also been reported. In a study done in 2007, the findings showed that noscapine induces apoptosis by increasing the activity of caspase 2, 3, 6, 8 and 9, DNA fragmentation in myelogenous leukemia cells of human and HL-60 cell line (15). In addition, with the doses used to treat vascular-spinal spasm diseases, the papaverine might induce apoptosis in endothelial cells and vascular smooth muscles (16). Codeine with 1-6 mM concentration leads to a reduction in proliferation (induced apoptosis) while a 0.5-8 mM concentration of this material results in a reduced percentage of cellular survival of Ramos cells in a dose-dependent manner (17).

However, because opium is a combination of 20 alkaloids and more than 70 different compounds (9), its effects are expected to be different from those of its components. Previous studies on opium and its effects on metabolism and body function are limited in number and scope. Considering the distinctive conditions of our country and problems of addiction to this drug, it seemed necessary to comprehensively investigate the effects of it on other cells and internal systems of the body. The present study was designed to determine the apoptotic effects of opium on AA8, AGS, Hela, HepG2, MCF7, N2a, PC12, and WEHI cell lines.

Materials and Methods

Cell Culture

The AA8, AGS, Hela, HepG2, MCF7, N2a, PC12,

Received May 3, 2016; **Accepted** September 23, 2016; **Published** September 30, 2016

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and WEHI cell lines were supplied (Iran Pasteur Institute, Tehran, Iran). The cell lines were cultured in Dulbecco's modified Eagle's Medium (DMEM) culture medium (Gibco Co., U.S) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco Co., U.S), 50 U/ml penicillin (Sigma-Aldrich Co., Germany) and 50 mg/ml streptomycin (Sigma-Aldrich Co., Germany). Then, the cell lines were maintained in an incubator at 37°C, which contained 5% carbon dioxide and 56% humidity. The Ethical Committee of Kerman University of Medical Sciences has approved the protocol study.

Adding opium to the culture medium

After attaining opium from anti-narcotics Police Department of Kerman (Iran), the gas chromatography-mass spectrometry (GC-MS) analysis showed that the opium was made of more than 30% alkaloids of different types (i.e. 16% morphine, 5.5% codeine, 4.4% thebaine, and 3.2% papaverine), non-alkaloid organic compounds and water. To create a stock solution (2.86 g/mL), based on the our previous studies (10,11,27) , powdered opium was dissolved in sterile PBS containing 10% ethanol and shaked at 200 rpm for 1 h at 56 ˚c. Afterwards, supernatant was obtained using centrifugation (2000 rpm, 10 minutes) and filtered through 0.2 μm filter. From 2.86 g/mL opium stock, 2.86 x 10^{-5} g/mL DMEM solution was prepared. The computations were done based on our previous study (11) and the pharmacologic doses and the effective concentrations of morphine upon Jurkat Cells and the assumption that opium contains 16% morphine. The 2.86 x 10^{-5} g/mL opium concentration in our previous study led to significant induction of apoptosis of Jurkat Cells (11) but it did not show significant effect upon the studied cell lines of the present study. As a result, the 2.86 x 10^{-4} g/mL opium concentration was selected for apoptosis induction.

Evaluating the cell lines survival

The survival extent of the cell lines was studied with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT is a yellow Tetrazolium salt dissolved in water reduced through succinate dehydrogenase enzyme inside the mitochondria of living and active cells, which turns into an insoluble purple-colored formazan compound. The color is solved in an organic solvent and its resulting color intensity in 570 nm wavelength matches that of the living cells.

After the growth and proliferation of the cell lines and then the separation of cells by Trypsin 0.25%, those cells with a relative number of 5 x 10^3 in each 96 plate wells were cultured in 200 µl of culture medium and incubated for 24 hours. Then, the medium of the cells was replaced by a complete medium of 2.86 x 10^{-4} g/ml opium concentration. The cells were incubated for 48 hours and then the medium of the cells was replaced with a new one.

MTT powder (Sigma-Aldrich Co., Germany) with 5 mg/ml was solved in PBS. Then 20 µl MTT solution was added to each well and the plate was incubated for 4 hours without any exposure to light. After incubation, the medium of the wells was emptied and 200 µl DMSO (Sigma-Aldrich Co., Germany) was added to each well. The optical density (OD) was read on an enzyme linked immunosorbent assay (ELISA) reader (Multiskan-MS, Germany) at 570 nm wavelength. The assay was repeated three times.

The Study of the Apoptotic Effects of Opium with Annexin V-FITC and Propidium Iodide (PI)

With the start of the apoptosis process, Phosphatidylserine, which is naturally produced in the inner half of the cell membrane, moves towards its external layer and becomes available. This is due to the disorder of ATP-dependent translocase enzyme or the activation of the other enzyme systems such as scramblase. Due to the fact that Annexin-V protein of high combination affinity can attach this phospholipid in the presence of calcium ion (Ca^{+2}), it is used as a marker for identifying apoptotic cells. PI is also a marker of the differentiation of necrotic and apoptotic cells which colors the nuclei of cells (18). First, 5 x 10^5 cells were poured in a tube containing 1 ml PBS. The cells were treated with opium for 48 hours. The control cells were also maintained in a medium without opium for 48 hours. Then, the kit guidelines were followed and 5µl Annexin and 5 µl PI were added (Sigma-Aldrich Co., Germany). The cells were analyzed by flow-cytometry (BD Co., U.S).

Statistical Analysis

The results were presented as mean ± standard errors (SE). After adding data to SPSS 16 for Windows (SPSS Inc., Chicago, IL, USA), the data was tested by one-way analysis of variance (ANOVA) and T-Test. The T-Test was used for the analysis and comparison of data between the two experimental and control groups. One-way ANOVA test was used to compare the cell lines and $P \leq 0.05$ values were considered to be significant.

Results

Effects of opium cytotoxic activity

All 8 cell lines were incubated for 48 hours with 2.86 x 10^{-4} g/ml opium concentration. Then, the cytotoxicity analysis of the effects of opium on the cell lines was done by MTT assay which showed that the cell lines manifested different levels of sensitivity to opium. The highest and lowest level of sensitivity to opium was respectively observed in AA8 and WEHI cells. The survival percentage of these cell lines was respectively 13.9% and 61.3%. Figure 1 shows the survival percentage of

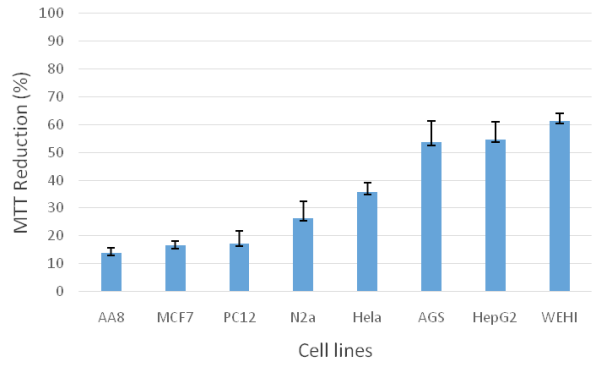

Figure 1. The percentage of cell lines viability after 48 hours incubation with opium concentration of 2.86 x 10^{-4} g/ml. The difference between the percentage of cell lines viability is statistically significant ($p<0.001$). MTT; 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide.

Table 1. Effect of opium on the induction of apoptosis and necrosis.

Cell type	% of total cell	
	Control	**Opium**
AA8		
Necrotic	3.46±0.71	3.58±0.53
late apoptotic	2.42±0.21	85.31±1.69*,#
Early apoptotic	0.64±0.08	1.17±0.27
Viable	93.47±0.42	9.93±1.95
AGS		
Necrotic	2.69±0.65	41.69±0.46#
late apoptotic	0.35±0.12	6.94±2.18
Early apoptotic	2.23±0.68	4.57±1.15
Viable	94.71±1.17	46.78±1.44
Hela		
Necrotic	2.38±0.6	36.36±2.46#
late apoptotic	1.33±0.17	26.22±1.14*,#
Early apoptotic	2.44±0.67	0.6±0.13
Viable	93.84±1.24	36.81±3.37
HepG2		
Necrotic	3.86±1.45	20.92±1.9#
late apoptotic	1.87±0.31	10.56±0.89*,#
Early apoptotic	0.83±0.1	0.44±0.1
Viable	93.44±1.82	68.06±2.50
MCF7		
Necrotic	2.74±0.6	4.12±0.97#
late apoptotic	2.97±0.47	86.17±1.94*,#
Early apoptotic	0.61±0.09	1.14±0.33
Viable	93.67±1.12	8.56±1.47
N2a		
Necrotic	5.27±0.61	4.02±0.54
late apoptotic	0.25±0.70	53.92±2.84*,#
Early apoptotic	0.13±0.14	10.28±2.35*
Viable	94.35±0.64	31.76±1.27
PC12		
Necrotic	3.14±0.22	7.31±0.49#
late apoptotic	1.17±0.19	82.51±1.19*,#
Early apoptotic	0.99±0.56	0.60±0.16
Viable	94.68±0.69	9.57±2.25
WEHI		
Necrotic	3.59±0.4	2.33±0.56
late apoptotic	0.52±0.03	5.96±1.34
Early apoptotic	2.45±0.36	31.49±2.79*
Viable	93.43±0.61	60.48±3.82

Cells were incubated for 48 hours without (control), or with 2.86×10^{-4} g/ml of opium. Each value (% cells) represents mean ± SEM from 3 independent FACS analyses. AA8 cell line (*#P<0.00), AGS cell line (#P<0.00), Hela cell line (*#P<0.001, #P<0.001), HepG2 cell line (*#P<0.003, #P<0.015), MCF7 cell line (*#P<0.000. #P<0.035), N2a cell line (*P<0.029. *#P<0.01), PC12 cell line (*#P<0.000.,*P<0.004). WEHI cell line (*P<0.004).

Late apoptotic*#, Early apoptotic*, Necrotic #.

cell lines after 48 hours of incubation of the cells with 2.86 x 10^{-4} g/ml opium, as determined by MTT assay.

Effects of opium apoptosis induction

The opium-induced apoptotic effects on the cell lines are shown in table 1. The results showed that opium concentration of 2.86 x 10^{-4} g/ml which has been treated for 48 hours significantly induced the apoptosis in certain cell lines (i.e. AA8, N2a and WEHI), apoptosis and necrosis in some others (i.e. Hela, HepG2, MCF7, and PC12), and only necrosis in the AGS cell line ($P<0.05$).

Discussion

The results of the present study showed that opium with 2.86 x 10^{-4} g/ml concentration treated for 48 hours significantly induced the apoptosis in certain cell lines (i.e. AA8, N2a and WEHI), apoptosis and necrosis in some others (i.e. Hela, HepG2, MCF7, and PC12) and solely necrosis in the AGS cell line. One of the reasons of individuals' inclination to use narcotics is the rumors about the therapeutic effects of opium on certain diseases although some people use narcotic drugs for fun or forgetting their personal and social problems (16, 19). As WHO reports show, 2.8% of Iranian adults are addicted to opium (20). Despite of prevalence and long background of opium usage, the studies concerning this matter are limited in number. However, there are a lot of studies concerning the effects of main components of opium such as morphine, codeine, papaverine and noscapine on the programmed death of different tissues and cells of the body (16, 21). Morphine induces apoptosis through FADD/P53 pro-apoptotic pathway of Jurkat Cells (22). Other associated studies found out that morphine caused induced expression of pro-apoptotic FAS Gene and facilitation of apoptosis through FAS Pathway (23, 24). Yin et.al (24) emphasized the apoptotic effect of morphine and stated that human peripheral blood lymphocytes influenced by morphine, represented higher levels of FAS expression. In other studies, they referred to the induction of the expression of FAS in T cell hybridomas of human lymphocyte caused by opioids. In a study (25) concerning the effects of morphine on cell proliferation, the results showed that morphine stops the proliferation of tumor cells in concentrations of higher than 10 micromoles but it induces apoptosis in concentrations of higher than 500 micromoles. In another study, the researchers found out that morphine induced different levels of apoptosis in HL-60 and A549 cell lines but it generated necrosis in

MCF7 (14). Our study also obtained the similar results. Opium induced apoptosis of different levels in some cell lines but it led to concurrent induction of apoptosis and necrosis in MCF7. The apoptotic role of some other primary derivatives of opium has also been reported. Noscapine reportedly induces apoptosis of cancer cells of the breast, lung, colon and ovary. It also acts as an anti-tumor drug through cessation of metaphase stage and the inhibition of cell divisions both of which induce apoptosis (26). In addition, the apoptotic role of papaverinein in aortic smooth muscle cells and coronary endothelial cells was observed in a concentration-dependent trend (16). Codeonine is the oxidized derivative of codeine which induces the apoptosis of leukemia HL-60 cell line in a concentration-dependent trend (21). Concerning the effects of opium on apoptosis, no more than the few studies which the authors of the present study have done, was found (10, 11, 27). The studies of AsadiKaram et.al (10, 27) concerning the effects of opium addiction on the apoptosis of liver, brain and ovary cells, and the results from the rats' testes showed that opium addiction induces the apoptosis of such cells. The results of the present study also showed that opium induces the apoptosis of HEPG2 (i.e. liver) and AA8 (i.e. ovary) cell lines which supports the results of our previous studies. In another study (11), Asadikaram et.al found out that opium has inductive effects of apoptosis and necrosis on Jurkat Cells and the apoptotic role of these cells is significant. In the present study, the results showed that the analyzed cell lines has less sensitivity to opium compared to the Jurkat Cells so that effective opium doses upon Jurkat Cell line (i.e. 2.86×10^{-5} g/ml for 48 hours) did not lead to significant apoptotic effects on the selected cell lines for analysis in our study. However, the higher dose of 2.86×10^{-4} g/ml opium treated for 48 hours caused significant apoptosis.

In the present study opium was used. Based on the fact that:

a) Opium contains more than 20 alkaloids and more than 70 different compounds (28).

b) There are different alkaloids receptor types: Mu (μ), Delta (δ), Kappa (κ), Nociceptin (ORL1)) , zetta (ζ) (with each-receptor subtypes: μ1, μ2, μ3 ; δ1, δ2 ;κ1, κ2 , κ3) (28).

Therefore; each cell may receive different message from different opium-alkaloids /and other opium-components, depends on the receptor subtypes, and consequently have diverse responses.

Furthermore, as described in the last paragraph of introduction section, so far there are few study conducted on biological effects of opium, therefore, it seems that more studies are necessary to fulfill the gaps in our knowledge.

The results of the present study suggested that based on the cell line, opium exerts different apoptotic and necrotic effects. Of our significant results was the determination of different sensitivity levels of diverse tissues to opium. Therefore, one could conclude that taking opium results in different levels of apoptosis in diverse tissues. It also causes conditions that lead to the disorder of some tissues and might possess therapeutic applications in certain other cases (e.g. unchecked growth of some cells) as suggested by our results.

Acknowledgements

We thank Professor Hamid Najafipour for his helpful scientific advice. This work is the product of Biochemistry MSc thesis of Mr Morteza Khaleghi.

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