Chronic Opium Treatment Can Differentially Induce Brain and Liver Cells Apoptosis in Diabetic and Non-diabetic Male and Female Rats

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It has been shown that some opium derivatives promote cell death via apoptosis. This study was designed to examine the influence of opium addiction on brain and liver cells apoptosis in male and female diabetic and non-diabetic Wistar rats. This experimental study was performed on normal, opium-addicted, diabetic and diabetic opium-addicted male and female rats. Apoptosis was evaluated by TUNEL and DNA fragmentation assays. Results of this study showed that apoptosis in opium-addicted and diabetic opium-addicted brain and liver cells were significantly higher than the both normal and diabetic rats. In addition, we found that apoptosis in brain cells of opium-addicted and diabetic opium-addicted male rats were significantly higher than opium-addicted and diabetic opium-addicted female, whereas apoptosis in liver cells of opium-addicted and diabetic opium-addicted female rats were significantly higher than opium-addicted and diabetic opium-addicted male. Overall, these results indicate that opium probably plays an important role in brain and liver cells apoptosis, therefore, leading neurotoxicity and hepatotoxicity. These findings also in away possibly means that male brain cells are more susceptible than female and interestingly liver of females are more sensitive than males in induction of apoptosis by opium.

Key Words: Addiction, Apoptosis, Brain cells, Diabetes, Liver cells

INTRODUCTION

Apoptosis or programmed cell death is known as a normal process in the development of the nervous system and aging. Apoptosis play a role in neurodegenerative diseases, including Alzheimer's and Parkinson's diseases [1]. Apoptosis is also a pathologic feature of the brain injury, certain inflammatory diseases of the brain and central nervous system infection, such as human immunodeficiency virus (HIV)-associated dementia [2]. Apoptotic effects of opioids such as morphine [3-7], heroines [8], codeine [9], in neurons has been demonstrated.

Opium is a narcotic analgesic drug which is originally obtained from the unripe seed pods of the opium poppy. Opium is used as the raw material for the synthesis of some medications such as morphine, noscapine, papaverine and codeine which contains 8∼17%, 1∼10%, 0.5∼1.5% and 0.7∼5% of opium, respectively [10].

Effects of opium on some biochemical parameters [11,12] and TGF-β [13] have been reported. Due to the lack of literature on the effects of opium we aimed this project to examine its effects on brain and liver cells apoptosis. Apoptotic activity of morphine [3-5] noscapine [14-17], codeine [9] and papaverine [18] has been shown in the *in vitro* and *in vivo* experimental systems. Morphine promotes Jurkat cells apoptosis [3] Morphine enhances the ex-

ABBREVIATIONS: HIV, human immunodeficiency virus; TGF-β, transforming growth factor beta; FasL, Fas ligand; STZ, streptozocin; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labeling; ANOVA, analysis of variance; EDTA, ethylene diamine tetra acetic acid; WHO, world health organization; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; MMPs, mitochondrial membrane potentials; SPSS, statistical package for the social sciences; NIH, national institutes of health; NO, nitric oxide; NF-κB, nuclear factor kappa B; TE, tris-EDTA; IAP, inhibitory apoptosis protein; ASK1, apoptosis signal-regulating kinase 1; JNK, c-jun NH2-terminal kinases.

pression of both Fas and Fas ligand (FasL), and induces macrophage apoptosis [6]. Fas (CD95)-induced hepatocyte apoptosis and cytotoxic activities of infiltrating neutrophils in the injured liver are two major events leading hepatitis [7]. Noscapine and papaverine are other important derivatives of opium [10]. Noscapine is commonly used as an antitussive agent in many countries. It binds stoichiometrically to tubulin, causes microtubule assembly and arrests mammalian cells in mitosis phase, and finally induces apoptosis [14,15]. Noscapine increases sensitivity of HCT116 colon carcinoma cells to apoptotic stimuli [17].

Papaverine is a vasodilator which is commonly used for the treatment of vasospasmic diseases such as cerebral spasm associated with subarachnoid hemorrhage, and in the prevention of spasm of coronary artery bypasses graft by intraluminal and/or extraluminal administration. Papaverine could damage endothelial and smooth muscle cells by inducing changes which are associated with events leading to apoptosis [18].

There are however, more than 20 alkaloids [19] and more than 70 components [20] in opium, thus, its effect on apoptosis and cell functions could hence be different from pure morphine, noscapine, codeine and papaverine.

Our previous findings on the effects of opium on biochemical parameters and TGF-β and also the influence of opium components on apoptosis encouraged us to investigate the effects of opium on apoptosis of brain and liver in male and female rats following repeated daily opium administration at a maximum tolerance dose (150 mg/kg). Due to the fact that, some people around the world believe that opium posses therapeutic properties on many disorders, particularly diabetes mellitus [11,12] in this study we designed a diabetic animal model to challenge this public believe on opium properties in an experimental manner.

METHODS

Materials

Opium was dedicated by anti-drug section of Kerman Police (Iran). Based on their information the origin of opium was Helmand in Afghanistan. Streptozocin (STZ) were purchased from Pfizer Company (AG, Zurich, Switzerland). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labeling (TUNEL) kit was purchased from Roche Diagnostic, Manheim, Germany. Proteinase K was from Roche, Germany [20 μ g/ml in phosphate-buffered saline (PBS)]. DNA extraction kit was from Cinnagen, Iran. Glucose oxidase kit was also prepared from Pars azemoon company (Tehran, Iran). All of other materials were of analytical grade and obtained from standard sources.

Methods

In this experimental study, 70 male and 70 female Wistar rats (weighting 250~300 gr) were entered into the study. Animals were kept on a 12 hours light-dark cycle and had freely accessed to food and water. All animals' procedures were in accordance to "guide for the care and use of laboratory animals (NIH US publication no. 85-23 revised 1985)". 20 of each group were used as control and 50 male and 50 female rats were used for inducing diabetes by injection of streptozocin (dissolved in sodium citrate buffer, pH 4.4) with a single dose of 60 mg/kg of the body weight

intravenously into the tail vein [21]. After 3 days, 0.5 ml blood was collected from orbit cavity by a thin heparinized tube. Plasma glucose was measured using glucose oxidase method and the animals with glucose level more than 250 mg/dl were regarded as diabetic [22]. The success rate of inducing diabetes was approximately 90%. 25 of the above animals along with 10 of the control (non-diabetic) animals were treated with a daily double dose (8 AM and 8 PM, ip) of opium for 8 consecutive days (Opium was dissolved in fresh saline). The protocol of opium treatment was as follow: Days one to five, 30, 60, 90, 120 and 150 mg/kg respectively and continued on 150 mg/kg for the consecutive three days (150 mg/kg was the maximum tolerable dose for animals). At day 9 after weight measurement, a single dose of 150 mg/kg opium was injected and after 3 hours animals were humanly killed by decapitation under ether anesthesia. The control groups received normal saline. All control animals survived during 14 days of experiment period. Overall 40% of STZ-diabetic rats survived during this period and there were no significant difference between males and females in this regard. Finally the liver and brain of 7 animals of each group were used for apoptosis assessment. The withdrawal signs in opium-dependent rats were observed from the 5th day of opium injection. These signs which were sometime observed for a short time before next injection were wet-dog shakes as the first sign, and then hyperactivity, irritability, head shakes, ptosis, and writhing.

Tissue preparation for histological studies

The rat brains and liver were rapidly removed and fixed at room temperature in phosphate-buffered saline (PBS; 0.1 M Sodium phosphate, 0.14 M NaCl, pH 7.4) containing 3.7% paraformaldehyde for 30 minutes. The tissues transferred to fresh 3.7% paraformaldehyde and incubated for 7.5 hours. Brains transferred to 75% ethanol and wash for 1 hour and repeated the wash using fresh 75% ethanol. This step was repeated by using of 95% ethanol and washing process was followed by 100% ethanol. The tissues transferred to xylenes and washed twice for 1 hour. The tissues embedded in molten paraffin wax (58°C) for 1 hour using new paraffin wax.

Apoptosis studies

The apoptotic nuclei DNA fragmentation (the final result of the apoptotic process), was evaluated by Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labeling (TUNEL) technique using the in situ cell death detection kit as described by manufacturer. Briefly, paraffin-embedded tissue sections (5 μ m), were treated with proteinase K (2 μ g/ml in PBS) at room temperature for 10 min and then soaked in PBS for 5 min. Sections were then incubated with 50 μ l terminal deoxynucleotidyl transferase and nucleotide mixture (TUNEL Label Mix) in a humidified chamber at 37°C for 1 hour in the dark. The sections were immersed in PBS. Slides were washed in PBS, and visualized with a fluorescent microscope (Micros Austria) using an excitation wavelength of 450~500 nm. Quantitative evaluation of apoptosis index was performed by manual counting of positively stained nuclei in 30 microscopic fields at ×100 magnifications.

Detection of brain cell apoptosis by gel electrophoresis

Gel electrophoresis is a simple method for detection of apoptosis. DNA extraction was performed according to the manufacturer's protocol. Briefly, a volume of 100 μl protease was added to 50 mg brain or liver tissue and was vigorously vortexed, and followed by addition of 5 μl of protease and then was incubated at 55°C for 2 hours. At the end of incubation time 400 μl of lyses solution was added, vortexed and homogenized for 15~20 minutes and followed by addition of 300 μl of precipitation solution and vortexing for 3~5 seconds and then centrifuged for 10 minutes at 1,200 g. The supernatant was discarded and 1 ml of washing buffer was added to the resultant pellet, 3~5 seconds vigorously vortexed and centrifuged for 5 minutes at 1,200 g. The washing step was repeated twice and at the end of second step washing buffer was discarded and the pellet was dried at 65°C for 5 minutes. Finally 50 μl of solvent buffer was added, gently vortexed and incubated at 65°C for 5 minutes, centrifuged for 30 seconds at 1,200 g. The resultant product is a biphasic solution which the upper layer is DNA product and the lower is the other materials. DNA was run on a 1.8% agarose gel electrophoresis at 5 V/cm in 0.5×TE buffer (Tris 10 mM; EDTA 1mM, pH 8.0) containing 10 μg/ml ethidum bromide.

Statistical analysis

All analysis was performed by SPSS (version 18; SPSS Inc.). For comparison of mean values between two groups, the student's t-test was used. To compare values between multiple groups, analysis of variance (ANOVA) was applied. All values are means±SEM. $p < 0.05$ was considered statistically significant.

RESULTS

The changes in blood glucose level and body weights are shown in Table 1. There is no significant difference between weights of control, opium-addicted (non-diabetic) and opium-addicted diabetic rats.

Our results showed that there was a significant difference in apoptosis as follow:

Brain

1. In male rats

The difference between normal (1.50%±0.373) and opium-addicted diabetic (31.80%±0.774) and opium-addicted non-diabetic (31.00%±0.830) was statically significant (p $<$ 0.001 and p $<$ 0.001 respectively), while there was not significant difference between normal (1.50%±0.373) and non-addicted diabetic (3.90%±0.526) groups (p= 0.62). There was also a significant difference between non-addicted diabetic (3.90%±0.526) and opium-addicted diabetic (31.80%±0.774) groups (p $<$ 0.001). Comparison of our data indicated that
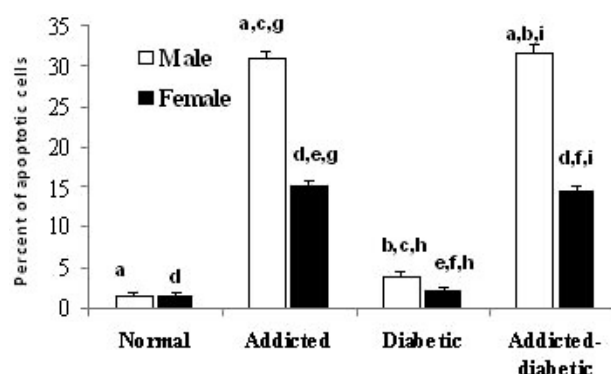
Fig. 1. Comparison of brain cells apoptosis in normal, opium addicted, diabetic and opium diabetic-addicted Wistar rats. Data are from 7 animals is presented in each group. In male rats: [a]Significant difference between normal, opium-addicted diabetic and opium-addicted non-diabetic (p $<$ 0.001 and p $<$ 0.001 respectively). [b]Significant difference between non-addicted diabetic and opium-addicted diabetic groups (p $<$ 0.001). [c]Significant difference between opium-addicted non-diabetic and non-addicted diabetic groups (p $<$ 0.001). In female rats: [d]Significant differences between normal, opium-addicted diabetic and opium-addicted non-diabetic groups (p $<$ 0.001 and p $<$ 0.001 respectively). [e]Significant difference between opium-addicted and non-addicted diabetic groups (p $<$ 0.001). [f]Significant difference between non-addicted diabetic and opium-addicted diabetic groups (p $<$ 0.001). Sex associated: [g]Significant difference between opium-addicted male and female (p $<$ 0.001). [h]Significant difference between non-addict diabetics male and non-addict diabetics female (p=0.006). [i]Significant difference between opium-addicted diabetic males and opium-addicted diabetic females (p $<$ 0.001).

Table 1. Blood glucose and weights of the study groups. Values are mean±SEM of 7 rats in each group

Parameters	Control	Diabetic	Diabetic-addicted	Addicted
Initial body weight (g)				
Male	280±20	283±22	282±17	278±19
Female	270±21	268±22	271±17	265±20
Final body weight (g)				
Male	287±19	268±24	270±27	280±21
Female	283±19	254±25	256±34	273±27
Initial blood glucose (mg/dl)				
Male	105.4±7.5	108.1±8.7	104.4±7.2	107.9±6.8
Female	107.4±8.5	106.0±6.8	103.4±7.9	105.3±5.8
Final blood glucose (mg/dl)				
Male	109.7±8.9	420.4±37.5*	390.9±43.1*	103.7±9.5
Female	105.8±9.9	450.9±57.5*	403.9±46.8*	108.7±8.5

*p $<$ 0.001 versus control.

a significant difference between opium-addicted non-dia-
betic (31.00%±0.830) and non-addicted diabetic (3.90%±
0.526) groups was also observed (p<0.001) (Fig. 1).

### 2. *In female rats*

We observed significant differences in female normal
(1.40%±0.0.267) and opium-addicted diabetic (14.36%±
0.256) and opium-addicted non-diabetic (15.20%±0.696)
groups (p<0.001 and p<0.001 respectively). The difference
is clearly significant in opium-addicted (15.20%±0.696) and
non-addicted diabetic (2.00%±0.05) groups when they were
compared (p<0.001). Interestingly, the non-addicted dia-
betic (2.00%±0.05) and opium-addicted diabetic (14.36%±
0.256) groups had also differences when they were com-
pared (p<0.001) (Fig. 1).

Liver

1. *In male rats*

The difference between normal (2.30%±0.448), opium-ad-
dicted diabetics (25.85±0.558) and opium-addicted non-dia-
betics (26.20%±0.800) was statistically significant (p<0.001
and p<0.001 respectively), but a significant difference was
not observed between normal (2.30% ±0.448) and non-ad-
dicted diabetic (2.02%±0.064) groups (p=0.649). There was
also a significant difference between non-addicted diabetics
(2.02%±0.064) and opium-addicted diabetics (25.85%±0.558)
(p<0.001).Our data analysis indicated a significant differ-
ence between opium-addicted non-diabetic (26.20%±0.800)
and non-addicted diabetic (2.02% ±0.064) groups (p<0.001)
(Fig. 2).
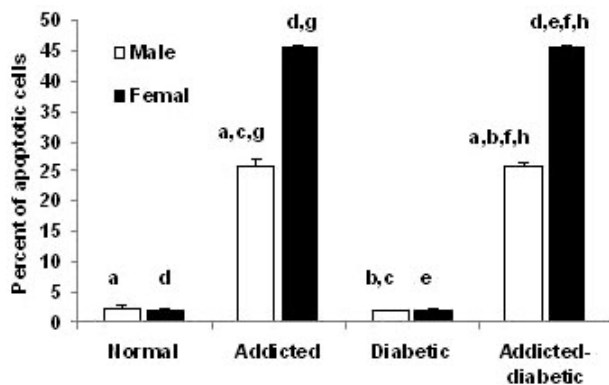
Fig. 2. Comparison of liver cells apoptosis in normal, opium
addicted, diabetic and opium diabetic-addicted rats. Data are from
7 animals is presented in each group. In male rats: [a]Significant
difference between normal, opium-addicted diabetics and opium-
addicted non-diabetics (p<0.001 and p<0.001 respectively). [b]Signi-
ficant difference between non-addicted diabetics and opium-addict-
ed diabetics (p<0.001). [c]Significant difference between opium-add-
icted non-diabetic and non-addicted diabetic groups (p<0.001). In
female rats: [d]Significant difference between normal, opium-addict-
ed diabetics and opium-addicted non-diabetics (p<0.001 and p
<0.001 respectively). [e]Significant difference between opium-addict-
ed and non-addicted diabetic groups (p<0.001). [f]Significant differ-
ence between non-addicted diabetics and opium-addicted diabetics
(p<0.001). Sex associated: [g]Significant difference between opium-
addicted male and female rats (p<0.001). [h]Significant difference
between opium-addicted diabetic females and addicted diabetic
males (p<0.001).

2. *In female rats*

We found significant differences in female normal (1.90±
0.314), opium-addicted diabetics (45.50±0.522) and opi-
um-addicted non-diabetics (45.00±0.715) (p<0.001 and p
<0.001 respectively). There is an obvious significant differ-
ence between opium-addicted (45.00±0.715) and non-ad-
dicted diabetic (2.09±0.08) groups (p<0.001). Obviously,
the non-addicted diabetics (2.09±0.08) and opium-addicted
diabetics (45.50±0.522) also showed significant difference
when they compared (p<0.001) (Fig. 2).

Sex associated apoptosis

1. *Brain*

Assessment of the effects of sex on apoptosis in our study
showed that there was a significant difference between opi-
um-addicted male (31.00±0.830) and female (15.20±0.696)
(p<0.001). There was also a significant difference between
non-addict diabetics male (3.90%±0.526) and non-addict di-
abetics female rats (2.00%±0.05) (p= 0.006). We also ob-
served that opium-addicted diabetic males (31.80±0.774)
have been significantly more affected by apoptosis than ad-
dicted diabetic females (14.36±0.256) (p<0.001) (Fig. 1).

2. *Liver*

In this study, we showed a gender dependent pattern of
the impact of opium on liver cells apoptosis. Evaluation of
the gender related effects of opium on apoptosis showed
that there was a significant difference between opium-ad-
dicted male (26.20±0.800) and female rats (45.00±0.715) (p
<0.001). We also showed that opium-addicted diabetic fe-
males (45.50±0.522) were more significantly affected by
apoptosis than addicted diabetic males (25.85±0.558) (p
<0.001) (Fig. 2).

Based on our findings apoptosis is following a differential
pattern in male and female rats, so that in male rats more
apoptotic brain cells but in females more apoptotic liver
cells were observed.

DISCUSSION

In the present study we found that apoptosis is induced
by opium addiction in diabetic and non-diabetic rat's brain
and liver. In addition brain cells of male rats are more sus-
ceptible to induction of apoptosis than female and interest-
ingly liver of females are more sensitive than males in in-
duction of apoptosis by opium. Some people in communities
believe that opium has positive therapeutic effects on some
disorders [12] including diabetes mellitus [11]. This is why
opium is prescribed by ordinary people and it makes an
excuse for consumers for explaining of its application.
Afghanistan, the eastern neighbor of Iran is the largest opi-
um producer and along the road of poppy transit to Europe.
Based on a WHO report, more than 2.8% of the adult pop-
ulation of Iran is opium-addicted [23].

Having chronic exposure to opiates lead to impairment
of impairs learning and memory [24] and opiate tolerance
and dependency have been suggested to induce a patho-
logical form of learning and memory [25] There are many
reports regarding induction of apoptosis by major compo-
nents of opium including; morphine, codeine, noscapine and
papaverine.

Chronic treatment with high doses of morphine induces

cell death via apoptosis in both neurons and glial cells [4]. Chronic, but not acute, intrathecal administration of morphine to rats produced neuronal apoptosis in the dorsal horn spinal cord [26]. In addition, chronic intraperitoneal treatment with morphine or heroin and heroin withdrawal induce apoptosis in the cerebral cortex of rats [5,27]. It has been shown that acute morphine (single dose) did not induced apoptosis but chronic administration of large morphine doses induced apoptosis in brain [4]. The morphological studies showed that chronic morphine treatment leading substantial injuries of brain. For example, chronic morphine treatment causes structural defects in cerebral cortex, hippocampus, and huge reduction in dendritic complexity and decreases denderitic growth in the cerebral cortex and hippocampus [28,29].

Life threatening effects of morphine are generally expected to happen at high doses, and this was our reason for selecting of maximum tolerance dose of opium (150 mg/kg) in the current study. Morphine enhances apoptosis of human umbilical vein endothelial cells (HUVECs) via enhancement of intracellular reactive oxygen species (ROS), leading reduced mitochondrial membrane potentials (MMPs) and also release of NO and activated NF-kappa B [30].

Buprenorphine which is a semi-synthetic opioid derivative commonly is used for treatment of heroin addiction can induce liver and kidney failure in consumers, possibly through direct mitochondrial toxicity [31]. Liver plays a major role in body defense against xenobiotics hence this organ was chosen to investigate in opium addicted situation. It has been reported that systemic disease, most notably liver disease, is common among fatal opioid toxicity cases [23]. Morphine-induced apoptosis is reported in SH-SY5Y cells through activation of JNK mitochondrial death pathway, and ROS signaling exerts its positive feedback regulation on JNK activity [32].

Papaverine is a vasodilator which indicated to induce apoptosis in vascular endothelial and smooth muscle cells [18].

Noscapine which serves as a tubulin-binding agent has also been demonstrated to facilitate apoptosis of a colorectal carcinoma cells [17].

Although there are huge amounts of reports regarding the effects of opiate derivatives on brain cells apoptosis in database, to our knowledge this is the first study on the effects of opium on brain cells apoptosis.

Although, gender differences our results showed that apoptosis in brain cells of addicted male rats were significantly higher than addicted female, but in liver cells of addicted female rats were significantly higher than addicted male. This finding is consistent with our previous studies on biochemical parameters [11,12], TGF-β [13] and many other reports on brain apoptosis and brain injuries [33,34], liver apoptosis [35] or injury [36]. It has been shown that females are more protected from cerebrovascular diseases than males. The underlying mechanisms which are involved in these sex differences remained unclear but exposure to gonadal hormones, particularly estrogen, has been through to play a major role [33,37] Apoptosis is believed to be triggered by intracellular signals and or extracellular by extrinsic death activators [1]. The responsible agents of activation of a specific triggering event in each pathway have very varied results in male or female animals or tissue, suggesting that intrinsic differences are mostly based on sex [33]. One of the differences regarding gender depending effects of opioid could probably be due to the variation in the individual tissues and also possibly differential interruption of sex hormone functions in male and female. It is also probable that the level of apoptosis such as inhibitory apoptosis protein (IAP) or products of bcl-gene groups are different in various gender and tissues; therefore, it is valuable to examine the level of these proteins in different genders. On the other hand, the expression of these regulators of apoptosis may have defects at the level of translation, transcription or even degrade due to the opium consumption.

Diabetes demonstrated that induce apoptosis in central nervous system and also in neuronal related tissues, including peripheral nervous system, hippocampus, and ganglions [22,38,39].

Increased mitochondrial ROS, has been reported in hyperglycemic liver cells, which in turn activates apoptosis signal-regulating kinase 1 (ASK1) and c-jun NH2-terminal kinases (JNK), increases serine phosphorylation of IRS-1, and decreases insulin-stimulated tyrosine phosphorylation of IRS-1 [40]. Based on vulgar believe, some diabetics individuals consume opium for reduction of diabetic defects [11]. These believe encouraged us to investigate the effects of opium on brain and liver apoptosis in diabetic rats. Although differential expression of genes in liver in diabetes were shown [41], but as it is clear from Fig. 1 there is no difference between diabetic and non-diabetic groups in this regard. Pathogenesis of hyperglycemia needs probably longer time from that we had in this study. The difference between groups may be attributed to the effect of withdrawal on apoptosis, however this is unlikely because the withdrawal time in this study was short as opium injection was repeated each 12 hours (see method section).

In summary, our results showed that opium addiction could possibly impose damage in brain and liver cells by inducing apoptosis. In addition herein we showed that brain cells of male rats are more susceptible to opium induced apoptosis than female, but liver cells of female rats are more susceptible to induction of apoptosis by opium than male. It is worth to note that some patients, in particular diabetics' individuals often consume opium at high doses for a long period of time [11] that could be deleterious for the patients' liver and nervous system.

ACKNOWLEDGEMENTS

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