Morphine sulphate induced histopathological and histochemical changes in the rat liver

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**Article info**

**Abstract**

In this study, the histopathological and histochemical changes due to chronic usage of morphine sulphate in liver were assessed in rats with both light and electron microscopes. Twenty male albino rats (Rattus norvegicus) (130–150 g) were included and divided into four groups. Normal saline (5 ml) was given orally as placebo in the control group (N=5). Morphine groups (N=5) received morphine orally at a single dose of 5 ml/kg/day for 10, 20 and 30 days (groups II, III and IV), respectively. Liver specimens from all groups were evaluated for histopathological and histochemical changes. Light microscopy revealed severe centrilobular congestion, portal fibrosis with bile ductal proliferation and an increased inflammatory infiltration and focal parenchymal necrosis. Histochemical study revealed a progressive depletion of general carbohydrates and an increase in total protein contents. These changes were confirmed at ultrastructural level, including the presence of accumulated lipid in the hepatocytes; deposits of a collagen-like fibrous material were seen in the space of Disse and a reduction in the number of endothelial cell fenestrations. Our findings pointed out the risk of increased lipid fibrosis and hepatic damage due to long-term use of morphine. Although opioids are reported to be effective in pain management, their toxic effects should be kept in mind during chronic usage.

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**1. Introduction**

Opioids are the most potent and effective analgesics available and have become accepted as appropriate treatment for acute, cancer and non-cancer chronic pain (Collet, 2001; Quang-Cantagrel et al., 2000). Morphine, which is commonly used for the treatment of severe pain, is metabolized essentially in the liver, gastrointestinal tract and kidneys (Stain-Texier et al., 1998). Long-term administration of an opioid drug for chronic non-cancer pain continues to be controversial (McCarberg and Barkin, 2001).

The central role of liver and kidney in drug metabolism predisposes them to toxicity injury. Every drug has been associated with hepatotoxicity almost certainly due to the pivotal role of the liver in drug metabolism. Hepatic metabolism is first and foremost a mechanism that converts drugs and other compounds into products that are more easily excreted and that usually have a lower pharmacologic activity than the portent compound (Tolman, 1998). A metabolite may have higher activity and/or greater toxicity than the original drug. These metabolic products are able to induce free radicals and/or bind with glutathione (GSH), the natural scavenger of superoxide radicals. Both GSH conjugation and its subsequent depletion cause accumulation of free radicals as well as morphine metabolites induce directly and indirectly cellular toxicity (Calignano et al., 1992) with enzymatic inactivation, DNA damage and/or lipid peroxidation (Cavallo et al., 2007). The present study was carried out to clarify the correlation between ultrastructural changes with histopathological findings with both light and electron microscope to show changes occurred in the organelles and inclusions of the liver cell post-application of the repeated administration of an addictive morphine drug on the rat liver, in a trial to elucidate the cause of the hepatotoxicity in narcotic addicts with special reference to its effect.

**2. Materials and methods**

2.1. I: experimental animals

Male albino rats (Rattus norvegicus) were used in the present study weighing (130–150 g). They were acclimatized to the laboratory conditions for 2 weeks. Animals were housed in standard home cages with proper ventilation, temperature, illumination “12 h dark–light cycle”. Rats were put on standard diet rodent chow and water was supplied ad libitum. We chose 4-week-old rats as an animal model, because prepubertal stage is much more sensitive than adult stage (Kondo et al., 2006). Animals were maintained and handled humanely in accordance with the guidelines.
of the animal experiments of South Valley University, Aswan, Egypt.

2.2. II: drug

MS® continu® tablets (MST) were used in this study, which were obtained from Medical Union Pharmaceuticals Co. “MUP”—Abu Sultan-Ismailla, Egypt, licensed by mundipharma AG Switzerland. Each tablet contains 30 mg of morphine sulphate dissolved in a physiological sterile saline (0.9%) to give 5 mg morphine sulphate dose orally given to rats by the gastric tube.

2.3. III: animal groups and treatment

The experiment was carried out on 20 rats that were divided into four equal groups (5 rats in each). Group I served as a control group, was given orally the drug carrier “sterile saline”. While the other three groups were orally given morphine sulphate tablets (MST) dissolved in a sterile saline in a dose level of 5 mg/kg/day after 10, 20 and 30 days (groups II, III and IV), respectively, this dose is equivalent to the human therapeutic dose. The choice of dose was according to Fleckell (1984) and Pavabash et al. (2006).

2.4. IV: specimen preparations

At the end of the experiment, animals of different groups were killed by cervical dislocation under diethyl ether anesthesia and the liver was removed. For light microscope preparations, livers were cut into small slices fixed in 10% formalin and Carnoy’s fluid, then dehydrated in gradual series of ethanol, embedded in paraffin wax and sectioned at 5 μm thickness. Slides were stained with hematoxylin and eosin and Mallory triple stain for histological examination. General carbohydrates were demonstrated using periodic acid Schiff’s technique (PAS). Total proteins were detected using the mercury bromophenol blue methods. For transmission electron microscopy, livers were immersed in 5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 3 h and post-fixed in 1% osmium tetroxide. After dehydration in gradual series of ethanol, the tissues were embedded in epon 812. Blocks with tissues were cut into semithin sections, then stained with toluidine blue and examined using a light microscope. Representative fields of semithin sections were selected. Ultrathin sections were stained with uranyl acetate and lead citrate then examined with TEM, in the electron microscope unit at South Valley University. Evaluation was limited to characterization of subtle lesions and abnormal cells, because quantitative analysis is impractical with electron microscopy. Twenty cells from each specimen were examined. Mitochondria, nuclei, rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER) of hepatocytes were evaluated using a scoring system (Table 2) (Ates et al., 2006; Mas et al., 2008). Twenty nuclei, 50 mitochondria, 20 rERs and 20 sERs were examined for each animal.

2.5. Statistical analysis

Results are expressed as mean ± SEM. Mann–Whitney U-test for histopathological scores and Student’s t-test for ultrastructure scores were used to analyze the significance of differences between groups. Correlation of histopathological and ultrastructural scores was assessed with Pearson correlation procedure. The differences were accepted as statistically significant when P<0.05.

3. Histological findings

In the section of the control rat liver (group I), the hepatocytes are arranged in strands with one or two spherical nuclei, sinusoids are occupied by blood cells. The cytoplasm of hepatic cells is slightly eosinophilic; the central vein has generally a circular outline (Fig. 1a). Morphine sulphate treatment produced hepatic necrosis, inflammation, fatty accumulation, and fibrosis after 10, 20, and 30 days. Necrosis, inflammation, fatty accumulation and fibrosis were significantly lower in group 1 when compared with other treated groups (Table 1). Degree of necrosis, inflammation, fat accumulation, and fibrosis were scored as 0: absent; 1: slight; 2: moderate; and 3: severe as described elsewhere, with small modifications (Hernandez-Munoz et al., 1997). Histopathological evaluation was performed twice in four sections per slide from all animals in each group (Table 2).

The histopathological examination of the 10 days treatment with 5 mg/kg/day morphine sulphate (group II) revealed remark-
able histological changes when compared with the control. The central vein exhibited remarkable dilatation and a phenomenon of invading infiltrative inflammatory cells was well manifested at the central vein (Fig. 1b). After 20 days morphine administration (group III), the hepatic acini were destroyed and some of the hepatocytes showed cytoplasmic vacuoles with degenerated nuclei; others were normal appearing cells and were binucleated. The blood vessel was enlarged and congested and the hepatic cells were highly damaged. Evidently, the more striking sign of the liver tissue damage was well discerned at the central vein. Moreover, the cell membrane of the hepatic cells was unrecognized and if some of them could exist they were ill defined and ruptured and the sinusoidal space lacked their familiar shape (Fig. 1c). Furthermore, after 30 days of morphine administration (group IV) several of the hepatocytes were fused together forming degenerated areas of destroyed cells that lost their normal characters and thus became necrotic areas. Also, nuclei of some hepatocytes were normal appearing cells and were binucleated. The degenerated nucleus; and if some of them could exist they were ill defined and ruptured, the cell membrane of the hepatic cells was unrecognized and the sinusoidal space lacked their familiar shape (Fig. 1c).

Table 2 summarizes the amount of general carbohydrates according to the intensity of stainability (+++, strong (+), moderate (++), and slight (+)) depleted. In control animals the cytoplasm of normal hepatocytes gave a strong positive reaction to PAS either in the form of red granules of different sizes, coarse and fine or diffused form (Fig. 2a). In morphine treated groups there is a gradual decrease in the amount of carbohydrate contents was noticed in all subgroups (Fig. 2b–d), generally the amount of carbohydrates fluctuated. The heterogenic distribution to PAS reaction was noticed in two neighboring areas, one containing an increased amount and the other showing a decreased amount.

4. Histochemical findings

4.1. General carbohydrates

Table 3 summarizes the amount of general carbohydrates according to the intensity of stainability (+++, strong (+), moderate (++), and slight (+)) depleted. In control animals the cytoplasm of normal hepatocytes gave a strong positive reaction to PAS either in the form of red granules of different sizes, coarse and fine or diffused form (Fig. 2a). In morphine treated groups there is a gradual decrease in the amount of carbohydrate contents was noticed in all subgroups (Fig. 2b–d), generally the amount of carbohydrates fluctuated. The heterogenic distribution to PAS reaction was noticed in two neighboring areas, one containing an increased amount and the other showing a decreased amount.

4.2. Total proteins

Table 3 summarizes the amount of total proteins according to the intensity of stainability with mercury bromophenol blue (Hg-BPB), total proteins were demonstrated by the presence of bluish

Table 1

<table>
<thead>
<tr>
<th>Histopathological scoring</th>
<th>10 days</th>
<th>20 days</th>
<th>30 days</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrosis</td>
<td>1.20 ± 0.15</td>
<td>1.92 ± 0.01</td>
<td>2.27 ± 0.18</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.44 ± 0.13</td>
<td>1.69 ± 0.11</td>
<td>2.30 ± 0.07</td>
<td>&lt;0.03*</td>
</tr>
<tr>
<td>Fat accumulation</td>
<td>1.25 ± 0.12</td>
<td>3.21 ± 0.11</td>
<td>4.07 ± 0.22</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1.40 ± 0.13</td>
<td>2.27 ± 0.18</td>
<td>4.27 ± 0.19</td>
<td>&lt;0.005*</td>
</tr>
<tr>
<td>Total</td>
<td>5.29 ± 0.53</td>
<td>9.09 ± 0.41</td>
<td>12.91 ± 0.66</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Organellie injury scoring

| Mitochondria             | 40.7 ± 1.3 | 60.5 ± 21 | 98.9 ± 2.1 | <0.001* |
| rER                      | 20.1 ± 0.8 | 40.1 ± 12 | 45.6 ± 2.1 | <0.001* |
| sER                      | 18.4 ± 0.8 | 40.2 ± 13 | 48.3 ± 1.6 | <0.001* |
| Nucleus                  | 16.9 ± 1.2 | 30.3 ± 2.1 | 36.3 ± 0.9 | <0.001* |
| Total                    | 106 ± 4.1 | 171 ± 47.1 | 229.1 ± 6.7 | <0.001* |

ER: endoplasmic reticulum.

Table 2

<table>
<thead>
<tr>
<th>Ultrastructural scoring</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Prominent cristae</td>
<td>1</td>
</tr>
<tr>
<td>Edematous mitochondrion</td>
<td>2</td>
</tr>
<tr>
<td>Collection of amorphous material</td>
<td>3</td>
</tr>
<tr>
<td>rER</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Dilatation</td>
<td>1</td>
</tr>
<tr>
<td>Irregular lamellar organization</td>
<td>2</td>
</tr>
<tr>
<td>Presence of focal breaks</td>
<td>3</td>
</tr>
<tr>
<td>sER</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Dilatation</td>
<td>1</td>
</tr>
<tr>
<td>Vascularization</td>
<td>2</td>
</tr>
<tr>
<td>Presence of large degenerated areas, myelin figures</td>
<td>3</td>
</tr>
<tr>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Irregular chromatin distribution (margination, clumping)</td>
<td>1</td>
</tr>
<tr>
<td>Increased heterochromatin</td>
<td>2</td>
</tr>
<tr>
<td>Degenerated nucleus</td>
<td>3</td>
</tr>
</tbody>
</table>

rER: rough endoplasmic reticulum; sER: smooth endoplasmic reticulum.
colorations. In control animal liver, it has been found that all the hepatocytes were characterized by a high concentration of total proteins. The reaction was given by nuclear structures (chromatin granules, nucleolus, and nuclear membrane) and the cytoplasm in the form of granules of different sizes or diffused perinuclear or peripheral in position, and particularly adjacent to sinusoids (Fig. 3a). In morphine treated groups (II, III and IV), there was a general increase in protein (stainability) content proportional to the period of treatment and this is more pronounced in the basement membrane, nuclear structure and degenerated areas and moderate in the cytoplasm compared to the control group (Fig. 3b–d).

5. Ultrastructural examination

Control hepatocytes were normally polygonal with oval-shaped nuclei and one or two nucleoli and the cytoplasm was crowded with organelles, particularly rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, ribosomes, mitochondria and glycogen particles. The hepatocytes are exposed on each side to the sinusoids, which are lined by a discontinuous layer of fenestrated endothelial cells. The endothelial cells are separated from the underlying hepatocyte surface by space of Disse is continuous with the sinusoidal lumen, thus of the hepatocyte that faces the space of Disse bears many irregular microvilli protruding in that space (Fig. 4a). In contrast, the hepatocytes of liver from the group treated for 10 days with morphine sulphate showed marked pathological alterations. These alterations represent an aspect of liver sinusoidal “capillarization” induced by defenestration. Along with these sinusoidal alterations, accumulated lipids in the hepatocytes were present. Also, a mesenchymal inflammatory reaction was present in the lobules, portal and perportal areas. Kupffer’s and sinusoidal endothelial cells were increased in the lobules (Fig. 4b). 20 days of morphine sulphate administration revealed severe and extensive appearance: hypertrophied nuclei with irregular nuclear membrane, and nucleolar fragmentations; atrophied mitochondria with ill-differentiated cisternae. The mitochondria changed their size and shape, i.e. they became polymorphous with the thickening of their matrix. The Golgi apparatus appeared obviously hypertrophied. A striking alteration in the rough endoplasmic reticulum and dilated smooth endoplasmic reticulum, massive cytoplasmic lipid inclusions in most hepatocytes were noted. All hepatocytes showed only a few scattered glycogen granules were observed among dilated smooth endoplasmic reticulum (Fig. 4c). After 30 days of morphine sulphate treatment, besides all the last pathological changes, marked reduction in endothelial fenestrations with alterations in their distribution was seen. These changes represent an aspect of liver sinusoidal “capillarization” induced by defenestration. Deposits of collagen-like fibrous materials were seen in the space of Disse, which will inhibit the exchange between the sinusoidal plasma and parenchymal cells, and ultimately cause a disorder of microcirculation and further progress of cirrhosis (Fig. 4d). The changes in ultrastructural scores between the three treated groups were nearly the same when compared with histopathological scores (Tables 1 and 2). This indicates that the current histopathological scoring system used to describe tissue injury in the present study successfully reflects organelle-based ultrastructural changes in hepatocytes.

6. Discussion

According to the present study, administration of morphine sulphate induced histopathological effects in the liver tissues. The most remarkable changes are the dilatation and a phenomenon of invading infiltrative inflammatory cells, which was well manifested at the central vein, the hepatocytes showed cytoplasmic vacuoles with degenerated nuclei. The blood vessel was enlarged and congested. Moreover, the cell membrane of some hepatic cells was unrecognized and ruptured and the sinusoidal space lacks their familiar shape. Furthermore, several hepatocytes were fused together forming degenerated areas of destroyed cells and became necrotic areas. Also, nuclei of some hepatocytes were completely deminished leading to an existence of damaged cells. The degree of histopathological damage became more serious with the time of the experiment.

These results are in agreement with the results of Maruta et al. (1997), who found that methamphetamine and morphine induced centrilobular vacuolation and diffuse eosinophilic changes of hepatocytes. These damages spread to the midzone of the liver and
partial necrosis of the centrolobe, and diffuse fatty degeneration were observed in the liver from 2.5 h to 18 h after the administration. While Mori et al. (2007) reported that the coadministration of methamphetamine and morphine increased subacute toxicity or lethality in rodents accompanied by the rupture of cells in the kidney and liver, there was an increase in poly (ADP-ribose) polymerase (PARP)-immunoreactive cells in the heart, kidney and liver.

It is widely reported that hepatic toxicity induced by morphine in rat is related to its metabolites rather than morphine alone (Misra et al., 2004; Atici et al., 2005; Pavavbav et al., 2006). These metabolic products are able to induce free radicals and/or binding with glutathione (GSH), the natural scavenger of superoxide radicals. Both GSH conjugation and its subsequent depletion cause a free radical accumulation as well as morphine metabolites induce directly and indirectly cellular toxicity with enzymatic inactivation, DNA damage and/or lipid peroxidation. These results may be explained considering that the reactive metabolites do occur only in the liver for oxidation by cytochrom P450 (Cavallo et al., 2007). Also, Pavavbav et al. (2006) reported that repeated morphine administration even at lower doses would induce oxidative stress in the liver, which may contribute to induction of apoptosis in hepatocytes. Zhang et al. (2004) reported that morphine caused a seriously oxidative stress in mice livers and hence caused hepatotoxicity.

We found also that treatment with morphine sulphate induced portal tract fibrosis with bile ductal dilatation and proliferation or clusters of inflammatory cells surrounding portal area. Evidently, the more striking sign of the liver tissue damage was well discerned at the portal area. The hepatic vein exhibited striking dilatation with thickened wall surrounded by an exuberant amount of the collagenous fibers. We attribute that the mechanism of the injury may be that the growth factors may be released into hepatic sinusoids to activate stellate cells and to initiate fibrogenesis. Similar results were reported by Reeves and Friedman (2002), who described hepatic fibrosis as the presence of excess collagen due to new fiber formation, laid down as part of the tissue repair response to chronic liver injury. They reported that the principal effecter of hepatic fibrogenesis is recognized as the hepatic stellate cell. In response to liver injury they undergo an activation process in which they become highly proliferative and synthesize a fibrotic matrix rich in type I collagen. Also, George et al. (2001) examined the effects of dimethylsulphoxide (DMN) that can cause fibrosis of the liver. They observed collagen fiber deposition, together with severe centrilobular necrosis, focal fatty changes, bile duct proliferation, bridging necrosis and fibrosis surrounding the central veins. A decrease in total protein and increase in DNA were also documented.

In the present study, obvious alteration in the histochemical results of liver cells of the rat treated with 5 mg/kg/day of morphine sulphate was noted. Carbohydrate was found to undergo a remarkable diminution in all groups. Such diminution exhibited time-dependent characteristics. This feature contrasted several reports which had shown that opioids can cause hyperglycemia (El-Sherif et al., 2002; Campos et al., 2001), parallel to the studies of Lelevich (2007) who studied the effect of acute morphine intoxication on the functioning of glycolysis in the rat liver. The most pronounced effect was the increased glucose level in blood and liver tissue. The elevated morphine dose did not change glycolytic...
enzyme activities, decreasing glucose and glucose-6-phosphate contents.

On the contrary, a good support is provided to the present results. In this regard, Abdel-Raheem et al. (1991) indicated that marked declines occurred in the liver, kidney and brain glycogen contents as consequences of administration of heroin at fixed doses to adult albino rats. In addition, Zahran (1994) found that heroin administration led to a duration and dose-dependent decrease of glycogen content in liver and kidney of rabbit. Such decrease was found to be also concomitant with marked hepatic and renal G-6-Pase declines. The results were confirmed by the decline of serum glucose levels and the marked elevation of GGT and GPT activities in serum. The possible interpretation of our results of carbohydrates depletion in the present study could be attributed to the toxication effects of morphine sulphate on the liver cells; under pathological condition the cells lost their capacity to metabolize glycogen normally.

It has been also noticed that treatment with oral morphine sulphate had exerted a gradual increase in the protein contents of the liver cells. Such increase exhibited time-dependent characteristics. Experimental studies have also supported toxic effects of chronic use of opioids on liver. Mori et al. (2007) reported that the coadministration of methamphetamine and morphine increased subacute toxicity or lethality in rodents, accompanied by the rupture of cells in the kidney and liver, and an increase in poly (ADP-ribose) polymerase (PARP), immunoreactive cells in the heart, kidney and liver. However, Hashiguchi et al. (1996) studied the central effects of morphine and morphine-6-glucuronide on tissue protein synthesis. They found that morphine and M6G suppress tissue protein synthesis through central mechanisms, mediated by opiate-induced respiratory depression in association with neural and hormonal alterations.

Also, we found that morphine induced remarkable ultrastructural alterations that give a good support to the previous findings performed with the light microscope. These alterations represent an aspect of liver sinusoidal “capillarization” induced by defenestration. A long with these sinusoidal alterations, accumulated lipids in the hepatocytes were present. Deposits of collagen-like fibrous materials were seen in the space of Disse, which will inhibit the exchange between the sinusoidal plasma and parenchymal cells, ultimately producing liver cell injury. The nuclei appeared hypertrophied, with nucleolar fragmentation and nuclear envelope clearly visible. The number of mitochondria decreased in all hepatocytes. The Golgi apparatus appeared obviously hypertrophied. A striking alteration in the rough endoplasmic reticulum dilated smooth endoplasmic reticulum. All hepatocytes showed only a few scattered glycogen granules were observed among dilated smooth endoplasmic. Similarly, Neubauer et al. (2001) reported that the process leading to liver fibrosis resembles the process of wound healing, including the three phases following tissue injury: inflammation, synthesis of collagenous and noncollagenous extracellular matrix components, and tissue remodeling (scar formation). During the establishment of liver fibrosis, the basement membrane components collagen type IV, entactin and laminin increase and form a basement-membrane-like structure within the space of Disse. The number of endothelial fenestrae of the sinusoids decreases. These changes of the sinusoids are called ‘‘capillarization’’ because the altered structure of the sinusoids resembles that of capillaries. At the cellular level, origin of liver fibrogenesis is initiated by the damage of hepatocytes, resulting in the recruitment of inflammatory cells and platelets, and activation of Kupffer’s cells, with subsequent release of cytokines and growth factors. The hepatic stellate cells seem to be the primary target cells for these inflammatory stimuli, during fibrogenesis, they undergo an activation process to a myofibroblast-like cell, which represents the major matrix-producing cell.

Giebeler et al. (2009) reported that the hepatocyte growth factor (HGF)/mesenchymal–epithelial transition factor (c-Met) system is an essential inducer of hepatocyte growth and proliferation. Their results showed that liver fibrosis is characterized by an excess of collagen fiber deposition, and it is known that Kupffer’s cells play an important role by immunomodulation of the toxic response (Rodriquez et al., 2008). Also, Trigueiro et al. (1993) showed with electron microscopic studying, a thickening of the sinusoidal wall related to endothelial cell hypertrophy and to fibrosis of the space of Disse. This was generally associated with basement-membrane-like material and hepatocyte microvilli flattening. It is concluded that oral morphine sulphate induces significant morphologic changes in the liver tissue (vesicular changes, fatty changes, chronic hepatitis, and cirrhosis), and the severity of these changes increases with time of treatment.

References
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