Aflatoxin B1 Induces Apoptosis in Rat Liver: Protective Effect of Melatonin

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Abstract

OBJECTIVES: A study of liver apoptosis after aflatoxin B1 (AFB1) administration and the effect of melatonin (MEL) was investigated in male rats.

METHODS: Five groups of 15 rats each were used: controls, MEL Soln-treated rats (MEL dose, 5 mg/Kg body wt), AFB1-treated rats (50 µg/Kg body wt), MEL Soln+AFB1-treated rats, and MEL micro-capsules (MEL-MC)+ AFB1-treated rats. After 8 weeks of treatment, biochemical measurements in liver homogenates and histopathological examination of liver sections of different groups using light and transmission electron microscope were done. The caspase-3 enzyme activity, apoptotic marker, was determined in liver tissues. Because hepatic antioxidants represent the major defence against toxic liver injury, and they act as anti-apoptosis. So, the levels of glutathione (GSH) and zinc (Zn) and the enzyme activities of glutathione reductase (GR), glutathione peroxidase (GSPx) and glutathione-S-transferase (GST) were determined. In addition, the levels of malondialdehyde (MDA), a lipid peroxidation product, and nitric oxide (NO) levels were measured.

RESULTS: The levels of caspase-3 activities in AFB1 group were significantly higher than control group. The apoptosis was associated with degenerative and necrotic changes in the hepatocytes. Concomitantly, the levels of MDA and NO in liver tissues were significantly increased while the levels of GSH, Zn and enzyme activities of GSPx and GR in liver tissues were significantly decreased in AFB1 group compared to their levels in controls. Caspase-3 activity was positively correlated with MDA while negatively correlated with GSH, GSPx and GR in rat livers treated with AFB1. The apoptotic rate was significantly reduced when MEL co-administrated with AFB1. In rats which received MEL with AFB1, the levels of MDA and NO in liver tissues were significantly reduced while GSH and Zn levels and GSPx, GR and GST activities were significantly increased compared to AFB1 group. When MEL-MC co-administrated with AFB1 appeared more effective in reduction of apoptotic rate as detected by decline of caspase-3 activities (inhibition 66.82%) and confirmed by histopathology.

CONCLUSION: AFB1 can lead to direct or indirect caspase-3 activation and consequently to apoptosis in rat liver. MEL treatment of rats could enhance hepatic antioxidant/detoxification system which consequently reduce the apoptotic rate and the necrobiotic changes in the liver. MEL-MC exhibited an efficient protective effect against AFB1. Thus, clinical application of MEL as therapy should be considered in cases of aflatoxicosis.
Abbreviations:
MEL: melatonin
MEL-MC: melatonin microcapsule
AFB1: aflatoxin B1
MDA: malondialdehyde
TEM: transmission electron microscope
NO: nitric oxide
GSH: glutathione
GSPx: glutathione peroxidase
GST: glutathione S-transferase
GR: glutathione reductase

Introduction

Apoptosis is a mode of programmed cell death phenomena and a principle of biology ‘it is better to die than to be wrong’. It is characterized by a series of typical morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells [1].

Caspase-3 is one of cysteine proteases which play a major role in the execution of apoptosis [2]. A number of genetic and biochemical studies suggest that caspase activation is essential for the occurrence of the apoptotic phenotype of cell death [3]. A variety of caspase substrates are involved in the regulation of DNA structure, repair and replication. Caspase-3 substrate cleavage has been observed under oxidative stress in different pathological conditions [4].

Aflatoxins are one of the most dangerous mycotoxins known, owing to their high toxicity to both animals and human. Aflatoxin B1 (AFB1), a metabolite of Aspergillus flavus, is a potent hepatotoxic and hepatocarcinogenic mycotoxin. One of manifestations of AFB1--induced toxicity is oxidative stress [5].

Recently, it is accepted that oxidative stress is an apoptosis inducer [6]. Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defenses [7]. The glutathione (GSH) redox cycle is an important component of the antioxidant machinery in cells. In normal cells, a primary defense against oxidative damage is provided by antioxidants such as GSH and the onset of apoptosis is associated with a fall in intracellular GSH in numerous cellular systems [8].

Melatonin (MEL, N-acetyl-5-methoxy tryptamine), the main secretory product of the pineal gland, is an antioxidant, scavenges the hydroxyl radical [9] and inhibits the production of nitric oxide (NO) by reducing NO synthase (NOS) [10]. Moreover, MEL has been shown to scavenge the peroxynitrite anion [11], and effectively reduces lipid peroxidation induced by a variety of toxicants [12]. MEL has a molecular weight of 232 and is both lipid [13] and water soluble [14], although its solubility in lipid is clearly greater [12]. MEL easily enters cells and sub-cellular compartments, a feature not shared by most antioxidants [15].

Recently, studies showed that MEL enters the nucleus where it protects DNA from oxidative damage, thereby decreasing the incidence of cancer [16]. Many carcinogenic substances including AFB1 damage DNA. The destructive effects of these agents on the genetic material involve a variety of different mechanisms but often free radicals are involved. Tan et al., [17] showed that DNA damage caused by the chemical carcinogen safrole was reduced by physiological and pharmacological levels of MEL.

Our aim was to investigate the liver apoptosis after AFB1 treatment and the effect of MEL as an anti-apoptotic. For this purpose, the feature of apoptosis was examined microscopically and biochemically by measurement of caspase-3 activity in rat liver homogenates. In addition, the oxidative stress indices, lipid peroxidation product (MDA) and nitric oxide (NO) and antioxidants, GSH and zinc, and enzymes, glutathione peroxidase (GSPx), glutathione reductase (GR) and glutathione S-transferase (GST) activities were determined in liver homogenates to clarify their roles in that liver apoptosis induced by AFB1. Moreover, MEL was utilized in a new preparation, MEL micro-capsules (MEL-MC), to produce a sustained release dosage form to prolong its biological half-life.

Materials and Methods

Chemicals

Thiobarbituric acid, reduced glutathione, oxidized glutathione, sodium azide, 1-chloro 2,4 dinitrobenzene, 5,5-dithio bis (2-nitrobenzoic acid), aflatoxin B1, melatonin, cumene hydroperoxide, NADPH, glutathione reductase, sodium sulphate, naphthylene-diamine dihydrochloride, sulphanilamide, aprotinin, diithiothreitol, HEPES (4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid), CHAPS (3-(3 chloramidopropyl dimethylammonio)-1-propane sulfonate) and sodium nitrite were all obtained from Sigma (St. Louis, M.O., USA). Ac-Asp-Glu-Val-Asp-p-nitroaniline, and p-nitroaniline were obtained from Calbiochem (Calbiochem-Novabiochem Corp., San Diego, CA, USA).

Animal treatment

Seventy five healthy male Sprague-Dawley rats (100–150g body wt) were purchased from Animal House of Faculty of Medicine, Assiut University. All animals were conditioned at room temperature at natural photo period for one week before the start of the experiment. A commercial balanced diet and tap water ad libitum were provided. Animals were divided into five groups (15 animals each) and
received the tested compounds by gastric intubation (in 0.1M NaHCO3, pH 7.4) daily for 8 weeks. The control group received vehicle only. The second group received MEL (dissolved in vehicle) at a dose 5 mg/Kg body weight [11]. The third group received AFB1 at a dose 50 µg/kg. The fourth group received MEL (dissolved in vehicle) co-administrated with AFB1. The fifth group received MEL micro-capsules (MEL-MC) + AFB1 at the same time. All treatments were given two hours before sunset, when MEL receptors were active. MEL-MC contain the same dose of solution form given to rats daily. Livers were excised immediately after rats killing. Species of liver were taken for histopathological examination. The rest of liver parts was homogenized in ice-cold 100 mM phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer fitted with a Teflon Plunger. Homogenates were centrifuged at 11,000 x g for 20 min and resulting supernatants were stored at -80°C.

Histopathological examination:
Specimens from liver were taken from all rat groups after scarification. They were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned at 5 micron and stained with hematoxylin and eosin stain (H & E stain). Then they were examined by light microscopy.

Preparation of semithin sections: Other specimens were fixed in glutaraldehyde (5%) and approximately 1 x 1 x 1 mm blocks were prepared. These blocks were washed in cacodylate buffer (0.1 M, pH 7.2) for 1–3 hr and then post fixed in 1% osmium tetroxide for 2 hr. After repeated washing in cacodylate buffer (4 X 30 min) and dehydration in ascending grades of ethyl alcohol up to 100% (30 min for every concentration), the specimens were placed in propylene oxide and then in pure epon. Semithin sections were obtained and stained with toluidine blue (T.B.) for 2 min at 80°C and examined by light microscopy.

Transmission electron microscope (TEM): Representative fields of semithin section were selected. Ultrathin sections (70 nm) were cut with diamond knife using a reichert OMVs ultramicrotome. They were mounted in copper grids and stained with uranyl acetate lead citrate stain [18]. The ultra-structural investigation was carried out with TEM (Joel Cx II).

Preparation of MEL in the form of microcapsules (MEL-MC):
The dosage form, MEL micro-capsule, was prepared in the Department of Pharmaceutics, Faculty of Pharmacy, Assiut University. Chitosan micro-par-
Zinc content in liver homogenates. Parts of livers were weighed into a digestion vessel and 2 ml concentrated nitric acid were added and then heated for 150°C to complete digestion. The digest was diluted to 5 ml with bidistilled water. Zinc concentrations were measured by atomic absorption spectroscopic method (Shimadzu atomic absorption/ flame spectrophotometers Model AD-620). The specific cathode lamp used was at 213.9 nm. Two determinations were made for each solution. The accuracy and precision of the analytical methods were tested with standard reference materials.

Statistical analysis

The data were presented as means ± standard error (SE). Differences between groups were determined using one way ANOVA followed by Student’s Newman-Keuls t-test. The level of significance was accepted with P<0.05. Prism computer program (graphPad version 3.0) is used for statistical analysis.

The percent inhibition (I%) in the mean values of caspase-3 was calculated as follows:

I% = mean AFB1+MEL value – mean AFB1 value x 100
mean AFB1+MEL value

Results

The levels of caspase-3 activities showed high apoptotic rate in AFB1 group than control group. The levels of MDA and NO in liver tissues were significantly increased while the levels of GSH and Zn and enzyme activities of GSPx and GR in liver tissues were significantly decreased in AFB1 group compared to levels in controls (Table 1). The levels of caspase-3 activities were positively correlated with MDA while negatively correlated with GSH, GSPx and GR in rat livers treated with AFB1 (Table 2). The apoptotic rate was significantly reduced when MEL co-administrated with AFB1. In rats received MEL with AFB1, the levels of MDA and NO in liver tissues were significantly reduced while GSH and Zn levels and GSPx, GR and GST activities were significantly increased compared to AFB1 group (Table 3). When MEL-MC co-administrated with AFB1 appeared more effective in reduction of apoptotic rate as detected by decline of caspase-3 activities (inhibition 66.82%) and confirmed by histopathology. The percent inhibition of caspase-3 activities in MEL+AFB1 and MEL-MC+AFB1 groups were 36.12% and 66.82% respectively.

Histopathological results

The liver of most cases in AFB1 group showed vacuolar degeneration (Fig. 1a), in which the cytoplasm of hepatocyte contains many vacuoles with irregular border. The vacuoles either appear empty or having a glycogen faintly stained fluid. The nuclei showed as a half moon appearance (Fig.1b). The nuclei of the hepatocytes became smaller in size, their chromatin were condensed as well as the cytoplasm of the cell (Fig. 1c). Many apoptotic bodies were seen in the kupffer cells of the liver (Fig.1d). The apoptotic cells could recognize also by TEM, in which the cells were smaller in sized with condensed cytoplasm and smaller electron dense nuclei (Fig. 2a). Frequently, apoptotic bodies were seen in the kupffer cells (Fig.2b). Some cases in group A showed hyperplasia of the bile duct with focal area of necrosis infiltrated with mononuclear cells (Fig. 2c). One case showed microscopic preneoplastic nodule in which larger hepatocytes were a typically arranged in concentric manner forming nodules (Fig. 2d). The liver in AFB1 + MEL group showed marked congestion in the sinusoids with slight vacuolar degeneration of the hepatocyte and minute foci of hepatic cell necrosis (Fig.3a). The cytoplasm of some hepatocytes contain faint stained fluid. Some hepatic cells showed dark

### Table 1. Alterations of caspase 3 activities, oxidative stress indices and antioxidants in the liver tissues of AFB1- and MEL-treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Controls</th>
<th>AFB1-treated group</th>
<th>MEL-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 (p-NA/hr/mg protein)</td>
<td>16.59±0.97</td>
<td>33.73 ± 1.14***</td>
<td>15.78 ± 0.99</td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>1.12 ± 0.09</td>
<td>2.08 ± 0.19***</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>NO (nmol/mg protein)</td>
<td>0.20 ± 0.05</td>
<td>0.45 ± 0.01***</td>
<td>0.15±0.04</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>30.10±1.40</td>
<td>23.10 ± 1.10***</td>
<td>34.61 ± 3.10</td>
</tr>
<tr>
<td>GSPx (nmol/min/mg protein)</td>
<td>301.50±10.50</td>
<td>252.3 ± 19.20*</td>
<td>399.70 ± 9.30***</td>
</tr>
<tr>
<td>GR (nmol/min/mg protein)</td>
<td>138.6±12.90</td>
<td>109.2 ± 2.60*</td>
<td>228.10±22.10**</td>
</tr>
<tr>
<td>GST (nmol/min/mg protein)</td>
<td>500.0±30.50</td>
<td>365.30 ± 28.50**</td>
<td>590.20 ± 26.00*</td>
</tr>
<tr>
<td>Zinc (µg/g dry Wt)</td>
<td>4.80±0.46</td>
<td>2.88± 0.21***</td>
<td>4.90±0.41</td>
</tr>
</tbody>
</table>

AFB1, aflatoxin B1; MEL, melatonin; NO, nitric oxide; MDA, malondialdehyde; reduced glutathione; GSPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-s-transferase. Values are means ± SE for 15 rats. P values are shown as *p<0.05, **p<0.01, ***p<0.001 vs controls. Other details are given in Materials and Methods.

### Table 2. Correlation of caspase 3 with oxidative stress indices and antioxidants in the liver tissues of AFB1-treated rats.

<table>
<thead>
<tr>
<th>Variables</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 3 vs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>+0.56</td>
<td>P&lt;0.001*</td>
</tr>
<tr>
<td>NO</td>
<td>+0.12</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>GSH</td>
<td>-0.67</td>
<td>P&lt;0.01*</td>
</tr>
<tr>
<td>GSPx</td>
<td>-0.51</td>
<td>P&lt;0.001*</td>
</tr>
<tr>
<td>GR</td>
<td>-0.48</td>
<td>P&lt;0.01*</td>
</tr>
<tr>
<td>GST</td>
<td>-0.11</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Zinc</td>
<td>-0.24</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

* significant
Table 3. Alterations of caspase 3 activities, oxidative stress indices and antioxidants in the liver tissues of rats treated with MEL and AFB1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AFB1-treated group</th>
<th>MEL+AFB1-treated group</th>
<th>MEL-MC+AFB1-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 (p-NA/hr/mg protein)</td>
<td>33.73 ± 1.14</td>
<td>24.78 ± 0.88***</td>
<td>20.22 ± 1.01††</td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>2.08 ± 0.19</td>
<td>1.50 ± 0.16***</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>NO (ng/mg protein)</td>
<td>0.45 ± 0.01</td>
<td>0.32 ± 0.02***</td>
<td>0.21 ± 0.02††</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>23.10 ± 1.10</td>
<td>29.30 ± 0.70***</td>
<td>35.70± 4.00</td>
</tr>
<tr>
<td>GSPx (nmol/min/mg protein)</td>
<td>252.3 ± 19.20</td>
<td>459.0 ± 27.00***</td>
<td>399.7 ± 19.30</td>
</tr>
<tr>
<td>GR (nmol/min/mg protein)</td>
<td>109.2 ± 2.60</td>
<td>147.3 ± 8.20***</td>
<td>239.00± 25.20††</td>
</tr>
<tr>
<td>GST (nmol/min/mg protein)</td>
<td>365.30 ± 28.50</td>
<td>758.0 ± 29.50***</td>
<td>690.20 ± 26.00</td>
</tr>
<tr>
<td>Zinc (µg/g dry Wt)</td>
<td>2.88 ± 0.21</td>
<td>4.12 ± 0.31**</td>
<td>3.91± 0.91</td>
</tr>
</tbody>
</table>

MEL, melatonin; AFB1, aflatoxin B1; NO, nitric oxide; MDA, malondialdehyde; MEL, melatonin; MEL-MC, melatonin microcapsule; reduced glutathione; GSPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-s-transferase. Values are means ± SE for 15 rats. P values are shown as *p<0.05, **p<0.01, ***p<0.001 vs AFB1 group and †p<0.05, ††p<0.01, †††p<0.001 vs MEL+AFB1 group. Other details are given in Materials and Fig. 1.

Fig. 1. Liver of AFB1 treated rats showing: (a) Vacuolar degeneration (H.&E. stain, 10x10); (b) Half moon or shrunk nuclei (arrows) in addition to vacuolar degeneration (V) and glycogen infiltration (G) (T.B. stain, 10x100); (c) Condensation of nuclear chromatin with smaller in the nuclear size (arrows) (T.B. stain, 10x100), and (d) Multiple apoptotic bodies in the kupffer cells (arrows) (T.B. stain, 10x100).
stained cytoplasm and nucleus represented early stage of apoptosis (Fig. 3b). The TEM of the hepatocytes revealed normal nuclear appearance with minimum degeneration of the cytoplasmic organelles and presence of minute cytoplasmic vacuoles (Fig. 3c). In the liver of AFB1+MEL-MC group, the congestion, degeneration and apoptosis were subsided (Fig. 4a). Only oedema in the Disse space with few kupffer cells proliferation were noticed (Fig. 4b). Mast cell reaction was frequently seen in the portal areas of the liver in this group (Fig. 4c). The TEM revealed normal ultrastructure appearance of the cytoplasm and nuclei of the hepatocyte (Fig. 4d). The examination of the liver in the control and MEL groups revealed normal histological appearance.

**Discussion**

The liver is the target organ for AFB1. Ingestion of this mycotoxin, is know to be capable of inducing acute poisoning, aflatoxicosis, and is believed to be implicated in the development of primary liver cancer [30]. AFB1 was shown to be converted into its epoxide and this derivative produces DNA adducts causing DNA strand breaks and point mutations [31]. Under this pathological condition, the active process of cellular self-destruction, apoptosis, may be occurred.
In the present study, the caspase-3 activity, an apoptotic marker, was significantly increased in liver tissues of AFB1-treated rats than controls. This finding was confirmed by histopathological examination of liver tissues of AFB1-treated rats. The morphological changes of apoptosis found in rat hepatocytes, in the current study, were previously described by Saraste and Pulkki, [1] which involve contraction in cell volume, condensation of the nucleus and the nucleus becomes fragmented. Finally, the cell itself fragments to form apoptotic bodies that are engulfed nearby phagocytes. Moreover, in the current study, the levels of MDA and NO were significantly increased while antioxidants (GSH, Zn, GR, GSPx, and GST) were significantly lower in liver tissues of AFB1-treated rats than controls. In addition, the levels of caspase-3 activities were positively correlated with MDA while negatively correlated with GSH, GSPx and GR in rat livers treated with AFB1. Similarly, Shen et al., [32] provided in vivo evidence that AFB1 can cause lipid peroxidation in rat liver. The authors suggested that oxidative damage caused by AFB1 may be one of the underlining mechanisms for AFB1-induced cell injury and DNA damage, which eventually lead to tumorigenesis.
Besides oxidative damage directly inflicted on DNA by free radicals, there are other indirect mechanisms by which radicals can cause destruction of the genome. As already mentioned, lipid peroxides enter the nucleus where they react with Fe$^{+2}$ to generate the alkoxyl radical which attacks DNA [33]. Also, intracellular calcium levels increase as a result of oxidative damage to membranes, calcium then enters the nucleus where it can activate nucleases which cause DNA strand breaks [34]. These marked increases in lipid peroxides levels after AFB1 could be contributed to the effect of AFB1 on the antioxidant defense system. Marked reductions in antioxidant enzymes activities and tissue GSH concentrations result in oxidative damage of tissues [35]. GSH depletion is sufficient for the onset of apoptosis in cellular systems, especially, in hepatocytes, has been addressed in several studies [36]. The significant decrease in the levels of GSH observed in liver tissues after AFB1 exposure causes decreased degradation of lipid peroxidation leading to their accumulation which amplifies the toxicity of AFB1.

Recently, Nakatani et al., [37] suggested that intracellular zinc interfere with apoptosis process possibly through the regulation of cellular redox potential involving GSH. Marini et al [38] showed that caspase-3 activity can be modulated by Zn$^{2+}$ and by the cell redox state. Also, Chai et al., [39] demonstrated that the Zn$^{2+}$ directly inhibits caspase-3 activation. The changes in the redox state can alter the association of Zn$^{2+}$ to enzyme antioxidants and consequently impair their activation or may affect zinc binding to intracellular stores, thus altering its free to bound ratio and/or its sub-cellular compartmentalization [40].

The redox state of the cells appears to be crucial parameter for the determination of the ultimate action of NO on cell multiplication and survival. Pro-apoptotic effects are often observed when NO reacts with superoxide to produce the highly toxic peroxynitrite under condition of oxidative stress. Peroxynitrite anion toxicity not only relates to its ability to initiate lipid peroxidation, but it also reduces mitochondrial respiration, inhibits the function of membrane pumps, depletes cellular GSH and damages DNA leading to activation of poly (ADP ribose) synthesis which causes cellular energy depletion [41].

It is well established that NO activates the transduction pathways leading to apoptosis. NO stimulates the expression of enzymes and transcription factors involved in apoptosis such as the tumor suppressor P53. The latter molecule transactivate the expression of pro-apoptotic genes, such as bax, and that of the cyclin-dependent kinase inhibitor P21, whereas it down-regulates the expression of the anti-apoptotic protein bcl-2 [42].

The histopathological investigation revealed that AFB1 induced marked hepatotoxic effects, in the form of degenerative and necrobiotic changes which were manifested by vacuolar degeneration as well as appearance of minute foci of necrosis. It also induced apoptosis of the hepatocytes in the most of the exposed cases, and only one case showed preneoplastic foci. The apoptosis was manifested by condensation of nuclear chromatin into defined masses that become marginated against nuclear membrane as well as formation of half moon shaped or round dense nuclear remnants. The cells were shrinkage and the cytoplasm was condensed. In addition, the appearance of many apoptotic bodies may be taken up by neighboring cells (kupffer cells). All these features were described as a morphological criteria of apoptosis by many investigators [43]. However, besides the lipid peroxidation and oxidative DNA damage induced by AFB1, it is mutagenic mycotoxin and could induce DNA damage in genomic DNA at different site [44, 5]. It is suggested that apoptosis in rat liver induced by AFB1 may be due its action directly on DNA and indirectly through oxidative stress and activation of caspase-3.

Souza et al., [5] reported that the oxidative stress is the principle manifestations of AFB1–induced toxicity which could be mitigated by antioxidants. Previously, many researchers investigated the effect of different antioxidants except MEL on AFB1. It is well established that MEL is a direct free radical scavenger and an indirect antioxidant. Melatonin’s antioxidant actions probably derive from its stimulatory effect on SOD, GSPx, GR and glucose-6-phosphate dehydrogenase and its inhibitory action on nitric oxide synthase [45]. Also, MEL acts to stabilize cell membranes, thereby making them more resistant to oxidative attack [16].

In rats group received MEL with AFB1, the levels of caspase-3 activities, necrobiotic changes and apoptosis were significantly lower than AFB1 group indicating that the apoptotic rate was significantly reduced after utilization of MEL with AFB1. In addition, the levels of MDA and NO in liver tissues were significantly reduced while GSH and Zn levels and GSPx, GR and GST activities were significantly increased compared to AFB1 group. Recently, Ortiz et al., [46] suggested that anti-apoptotic action of MEL is one of the mechanisms by which it protects neuronal cells from neurotoxic insults. Kim et al., [47] suggested that MEL pretreatment inhibits radiation-induced apoptosis and MEL exerts its radio-protective effect by inhibition of lipid peroxidation and without any involvement of the P53/P21 pathway. Also, Atroschi et al. [48] found that MEL inhibited the lipid peroxidation induced by mycotoxin in vitro.
Melatonin’s ability to protect nuclear DNA from oxidative damage was reported [16]. Intracellularly, MEL may not be distributed uniformly. When pharmacological doses of MEL were given to rats, highest concentrations of MEL occurred in the nuclei of cells [49]. Finnachiara and Glikin [50] examined the sub-cellular distribution of labeled MEL and detected MEL in both the cytosol and nuclei. The apparent quantities of MEL in the cytosol and nuclei were shown to be fluctuated with the stage of the cell cycle. On the basis of their finding, Finnachiara and Glikin [50] speculated that MEL has a variety of functions within cells and assumed that one action may be the protection of macromolecules from free radical damage. Cuzzocrea et al., [51] speculated that melatonin’s protective effects were a consequence of its ability to reduce NO formation and scavenge peroxynitrite anion and associated oxidants. The MEL induced suppression of NOS activity is believed to be a consequence of the binding of calmodulin by MEL [52]. NOS is a calmodulin-activated enzyme and by binding calmodulin MEL may limit its availability for this function [53]. With a drop of NO synthesis, the formation of the peroxynitrite anion is curtailed, and the potential oxidative damage resulting from this latter molecule is averted [41].

The significant elevation in the levels of enzyme antioxidants, GR, GSPx, and GST activities in liver tissues of MEL+AFB1-treated rats was previously shown when MEL administrated alone in our study and other studies [16,45,54]. More definite proof of the interaction of MEL with the genome has come from the work of Steinhalber et al., [55]. The significant rise in enzyme antioxidant activities after MEL administration indicated that this action of MEL may be mediated via a nuclear MEL receptor with a subsequent action on gene expression.

When MEL was given orally to animals, rapid elimination from blood (a short half-life, 24 min) was reported [12]. So, MEL loaded micro-capsules were prepared to decrease drug dissolution, to prolong half life of the drug, to give more efficient utilization and to maintain a constant plasma level of the drug [56]. In the present study, we used MEL in the form of micro-capsule to prolong its half life to be more efficient as antioxidant. In comparison between the actions of MEL and MEL-MC on AFB1 toxicity, it appeared that MEL-MC is more effective in reduction of necrobiotic changes, apoptosis and caspase-3 activities and NO levels and elevation of GR activities. The inhibition percent of MEL-MC on caspase 3 was about two-fold than MEL+AFB1 group. This effect of MEL-MC shown on apoptotic rate in rat liver is most likely due to increase its half life to be able to increase reduced GSH by increase GR activities and reduce of NO levels.

In conclusion, our study shows that an increase in apoptotic rate in liver of rats treated with AFB1 is associated with biochemical disturbances in oxidant / antioxidant balance system which may be ultimately interlinked in the pathogenic network of the AFB1 toxicity. MEL is beneficial in cases of aflatoxicosis to inhibit liver apoptosis by correcting the disturbance of oxidant/antioxidant balance system and hence ensures early and complete recovery from AFB1 toxicity and thereby decreases incidences of liver damage. MEL, as a drug (particularly, MEL-MC), may be given to human in endemic regions of the world with AFB1-induced pathology to reduce an incidence of liver damage. A better knowledge of the regulation and execution of apoptosis may be potentially excellent target for diagnosis and therapeutic intervention in AFB1induced liver dysfunction. Further study for mutations in apoptosis related genes may be important to refine prognosis in liver cancer.

REFERENCES

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