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Scientific Paper

MELATONIN INHIBITS BENZENE-INDUCED LIPID PEROXIDATION IN RAT LIVER

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We studied the antioxidative role of melatonin against benzene toxicity in rat liver. The inhibition of mitochondrial and microsomal lipid peroxidation differed between 24-hour (single-dose), 15-day, and 30-day treatments. Inhibition of mitochondrial lipid peroxidation was the highest after the single dose of melatonin, whereas highest microsomal inhibition was recorded after 30 days of melatonin treatment. No significant difference was recorded between 15-day and 30-day treatments. Cytochrome P_{450}2E1 (CYP_{450}2E1) activity declined after the single-dose and 15-day melatonin treatment in the benzene-treated group, but it rose again, though not significantly after 30 days of treatment. Liver histopathology generally supported these findings. Phenol concentration in the urine samples declined in melatonin and benzene-treated rats. Our results show that melatonin affects CYP_{450}2E1, which is responsible for benzene metabolism. Inhibition of its metabolism correlated with lower lipid peroxidation. In conclusion, melatonin was found to be protective against lipid peroxidation induced by benzene.

KEY WORDS: *CYP_{450}2E1, GSH, histopathology, mitochondria, microsomes, phenol, urine*

Benzene has widely been used as a general purpose industrial solvent. However, it is now used principally as an intermediate in the synthesis of other chemicals. Epidemiological studies have linked occupational exposure to benzene with a variety of leukaemias in humans (1). It has been established that benzene needs to be metabolised by a hepatic cytochrome P_{450}2E1 (CYP_{450}2E1) to manifest its cytotoxic and genotoxic effects (2-4). Subsequent secondary activation of its metabolites by myeloperoxidase (MPO) present in the bone marrow results in the production of xenotoxic quinines and reactive oxygen species (ROS). The latter account for most of benzene toxicity (5).

Melatonin (N-acetyl-5-methoxytryptamine) has for long been associated with circadian rhythm. Recently however, Reiter (6) described an intriguing antioxidant property of melatonin. It protected against free radical-induced damage in rat liver by maintaining or increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (7-9).

Melatonin eliminated hydroxyl radical, singlet oxygen, hydrogen peroxide, peroxyl radical, and hypochlorous acid through its direct scavenging action (10-14). It was found to protect against the toxicity of alpha-naphthylisothiocyanate (15), acetaminophen (16), irradiation (17), and arsenic (18). In addition to liver, beneficial effects of melatonin have been observed in the skin (19), cerebral ischaemia (20), and ocular diseases (21).

An earlier report from our laboratory (22) showed that lipid peroxidation induced by benzene in rat liver and kidney oscillated between circadian rhythms. We speculated this could be because of melatonin.

This study was therefore performed to establish the influence of melatonin on lipid peroxidation in benzene-treated rats.

MATERIALS AND METHODS

Animals, benzene exposure, and melatonin treatment

Sixty three-month-old male Wistar rats were procured from the animal facility of Jamia Hamdard, New Delhi. They were maintained in the animal house of the Department under 12 h dark and light cycle. Each rat was offered pelleted food (Golden Feeds, New Delhi) and tap water *ad libitum*. Twenty male rats of equal, average body mass were selected for either of a 24-hour, 15-day, or 30-day experiment. The rats were further divided into four groups of five animals. Group A consisted of rats receiving benzene (CDH, Mumbai, India) alone. Group B consisted of rats receiving melatonin (Sigma Chemical Company, St. Louis, MO, USA) and benzene. Group C consisted of rats receiving melatonin alone. Group D were injected 0.2 mL olive oil (CDA, Mumbai, India) only and served as controls.

Benzene was administered to group A in the morning hours. Selection of dose was based on our previous studies on benzene toxicity, where the same dose and same method of administration were used (22). Rats received 0.20 mL of a benzene solution (2 % in olive oil) per 100 g of body mass. It was injected intramuscularly as a single dose, on each alternate day for 15 days, or on each alternate day for 30 days.

Melatonin was administered to group B at the dose of 10 mg kg^{-1} body mass 60 min before administration of benzene, which was administered at the rate described for group A. Group C received melatonin only in the same dose as the rats of group B. Melatonin was always administered in the morning hours.

The experimental protocol was approved by the Institutional Ethics Committee.

Tissue collection and sample preparation

Urine samples were collected from each rat through metabolic cages after 24 h, 15 days or 30 days of melatonin and/or benzene treatment. They were kept frozen till analyses for phenol. Liver samples were collected from each rat after sacrifice, blotted dry with filter paper and stored at -20 °C till analysis. The maximum storage time was one week. Ten-percent (w/v) homogenates were prepared using a Potter-Elvehjem homogenizer according to the method described for determination of malondialdehyde, reduced glutathione, and $CYP_{450}2E1$ in liver samples (27).

Phenol

Phenol was estimated in the urine samples using the amino antipyrine method of Dannis (23). Pure liquid phenol and amino antipyrine were procured from CDH, Mumbai, India. The absorbance was recorded at 510 nm using a spectrophotometer (Systronics, Ahmedabad, India).

Lipid peroxidation

Lipid peroxidation in the liver was determined by measuring mitochondrial and microsomal malondialdehyde following the method of Jordan and Schanman (24). Microsomes were separated using an ultracentrifuge (Sorval, Newtown, CT, USA) following the method by Schenkman and Cinti (25). Thiobarbituric acid-reactive substances were measured at 532 nm using a spectrophotometer (Systronics, Ahmedabad, India). 1,1,3,3 tetramethoxypropane (Sigma, USA) was used as the standard. Thiobarbituric acid was purchased from Sigma, USA.

Reduced glutathione

Glutathione (GSH) was determined in the liver using the Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid), Sigma, USA]. Sulphosalicylic acid was used for protein precipitation. Absorbance was recorded at 412 nm using a spectrophotometer (Systronics, Ahmedabad, India).

$CYP_{450}2E1$ activity measurement

CYP2E1 activity in microsomal preparations was estimated spectrophotometrically using the method of Koop (27). Briefly, the reaction mixture consisted of 0.2 g L^{-1} of microsomal protein, 0.1 mmol L^{-1} of potassium phosphate, pH 6.8, and of 1 mmol L^{-1} of p-nitrophenol. Samples were incubated at 37 °C for 3 min prior to the addition of NADPH to start the reaction. After 10 min, the reaction was stopped with 1.5 mol L^{-1} perchloric acid. Absorbance was measured at 510 nm. All these chemicals were procured from Sisco Research Laboratories, Mumbai, India.

Protein measurements

Protein content in the liver samples was measured applying the method of Lowry et al. (28). Bovine serum albumin (BSA) was procured from Sigma, USA.

Histopathological observations

Small pieces of liver collected from the mid liver lobe of all treated and control rats were fixed in 10 % neutral formalin, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Six-micrometer thick paraffin sections thus prepared were stained with hematoxylin and eosin and examined under light microscope (Nikon, Tokyo, Japan). Formalin, ethyl alcohol, paraffin, xylene, hematoxylin, and eosin were procured from Sisco Research Laboratories, Mombai, India.

Statistical analysis

The data were expressed as mean±SEM. Statistical evaluations were performed by the analysis of variance (ANOVA) (29). $P<0.05$ was considered as statistically significant.

RESULTS

Acute exposure to benzene significantly induced lipid mitochondrial and microsomal peroxidation in rat liver. GSH levels increased after acute treatment, but they dropped significantly after the 15-day and 30-day exposure. CYP_{450}2E1 activity increased after the single benzene dose (24 h), but decreased after 15 days and 30 days (Table 1).

Melatonin pre-treatment inhibited mitochondrial and microsomal lipid peroxidation in the liver of benzene-treated rats (Table 1). GSH levels increased in the liver of melatonin and benzene-treated rats (Table 1). CYP_{450}2E1 activity fluctuated decreased after 24 h, further decreased after 15 days, but improved after 30 days of melatonin administration to benzene-treated rats (Table 1). These observations are supported by the results on urinary phenol concentrations. It decreased after melatonin treatment for different durations (Figure 1).

No significant histopathological differences were observed between the groups of melatonin and benzene-treated rats. However, variation did occurr in rats treated with benzene alone. Benzene caused a

Table 1 *Effects of melatonin on lipid peroxidation in the liver of rats treated with benzene*

Group	Treatment	Cytosolic MDA / nmol mg^{-1} protein			Microsomal MDA / nmol mg^{-1} protein			GSH / µg g^{-1} wet liver			CYP2E1 / µg mg^{-1} microsomal protein		
		24 h	15 days	30 days	24 h	15 days	30 days	24 h	15 days	30 days	24 h	15 days	30 days
A	Benzene	0.790± 0.047^{NS} (0.821 to 0.75)	0.214± 0.018^{NS} (0.23 to 0.19)	0.268± 0.009^{NS} (0.289 to 0.235)	0.027± 0.0015^{NS} (0.031 to 0.022)	0.038± 0.0023^{NS} (0.045 to 0.030)	0.122± 0.0019^{NS} (0.128 to 0.117)	0.827± 0.0134* (0.87 to 0.79)	0.130± 0.0137^{NS} (0.175 to 0.095)	0.141± 0.012^{NS} (0.15 to 0.098)	209.0± 28.85^{NS} (282 to 106)	152.0± 19.911^{NS} (201 to 88)	93.2± 7.98^{NS} (125 to 75)
B	Melatonin + Benzene	0.530± 0.191* (0.585 to 0.47)	0.133± 0.002*† (0.142 to 0.125)	0.163± 0.004*† (0.181 to 0.158)	0.018± 0.011*† (0.07 to 0.002)	0.028± 0.0109*† (0.07 to 0.005)	0.177± 0.0179^{NS}† (0.23 to 0.126)	0.966± 0.151*† (1.4 to 0.53)	0.330± 0.0537*† (0.5 to 0.15)	0.368± 0.0499*† (0.53 to 0.19)	192.0± 20.30^{NS}† (251 to 127)	25.8± 5.5242*† (43 to 8)	74.0± 11.38*† (102 to 29)
C	Melatonin	0.410± 0.006^{NS} (0.432 to 0.390)	0.446± 0.255* (1.58 to .148)	0.159± 0.011* (0.21 to 0.14)	0.0156± 0.0077* (0.05 to 0.004)	0.022± 0.006^{NS} (0.051 to 0.009)	0.104± 0.007^{NS} (0.135 to 0.083)	1.916± 0.164* (2.53 to 1.5)	0.402± 0.0317^{NS} (0.45 to 0.3)	0.33± 0.037* (0.42 to 0.185)	189.0± 21.74^{NS} (252 to 112)	97.0± 7.11^{NS} (125 to 79)	98.4± 5.156* (83 to 50)
D	Control	0.130± 0.010 (0.35 to 0.28)	0.122± 0.0030 (0.129 to 0.11)	0.175± 0.019 (0.25 to 0.13)	0.012± 0.006 (0.04 to 0.003)	0.006± 0.001 (0.012 to 0.001)	0.008± 0.001 (0.015 to 0.004)	1.660± 0.366 (2.08 to 0.1)	0.586± 0.064 (0.75 to 0.38)	0.311± 0.040 (0.4 to 0.15)	51.4± 5.17 (68 to 37)	53.4± 5.17 (68 to 37)	55.4± 5.17 (68 to 37)

Results are expressed as mean±S.E. (n = 5)
** Denotes values significantly different from control rats*
† Denotes values significantly different from benzene-treated rats
NS denotes non-significant differences
All values are significant at $p<0.05$
Values in parenthesis indicate the range.
MDA - malondialdehyde
GSH - glutathione

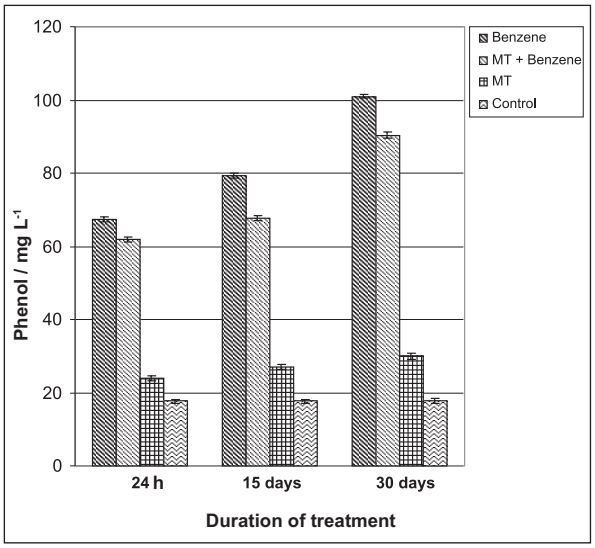

Figure 1 *Effect of melatonin (MT) on urine excretion of phenol in benzene-treated rats*

massive necrosis in the rat liver (Figure 2). However, in melatonin and benzene-treated rats no centrilobular necrosis was recorded (Figure 3). Surprisingly, peripheral (focal) necrosis was observed in the liver of melatonin-treated rats (Figure 4).

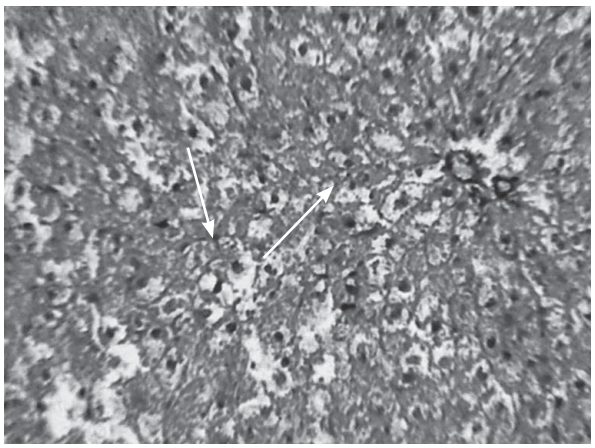

Figure 2 *Transversal section of the liver of a benzene-treated rat shows the development of massive necrosis in the centrilobular region (magnification x100).*

DISCUSSION

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine known to be involved in the biochemical regulation of the circadian rhythm and other biological functions (30, 31). It is also synthesised in extra pineal sites such as retina, Harderian glands, gut, ovary, testes, bone marrow, lens, and skin (32, 33). In mammals, melatonin is metabolised either directly

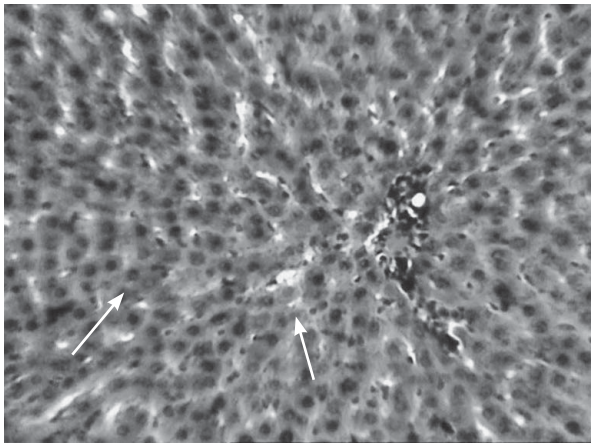

Figure 3 *Transversal section of the liver of a melatonin and benzene-treated rat shows no centrilobular necrosis. However, high mitotic activity was observed. The number of Kupffer cells was increased (magnification x100).*

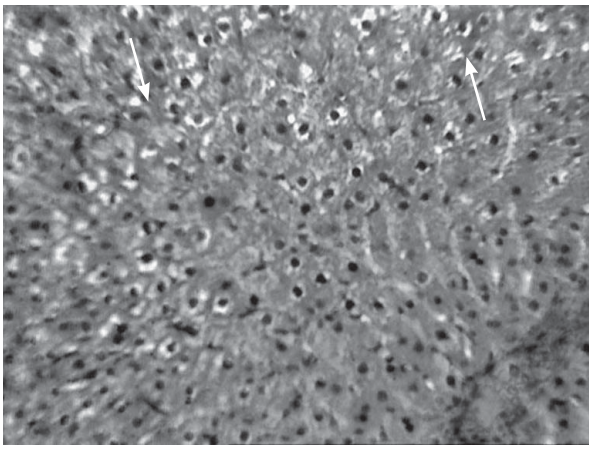

Figure 4 *Transversal section of the liver of a melatonin-treated rat shows focal necrosis (at the periphery of the lobule). No other pathological changes were observed (magnification x100).*

at the site of production or in the liver (for circulating melatonin) through complex pathways. Thus through side chain changes melatonin can be transformed into 5-methoxyindole acetic acid or 5-methoxytryptophol (34). Alternatively, by indoleamine 2,3-dioxygenase through the cleavage of the pyrrole ring, it can form N^1-acetyl-N^2 –formyl-5-methoxy-kynuramine (AFMK) (35). Reactive oxygen species are known to mediate in the oxidation of melatonin to AFMK. We believe that ROS may also cause focal necrosis in the liver.

Melatonin is well known to scavenge oxygen-free radicals and to inhibit, *in vitro* and *in vivo,* lipid peroxidation generated by ethyl alcohol (36), carbon tetrachloride (37), and paraquat (38). This is the first report that shows that melatonin also inhibits

benzene-induced lipid peroxidation. All doses of melatonin administered to benzene-treated rats were found to attenuate the increase in thiobarbituric acid reactive substances (TBARS) concentrations observed at progressing stages of liver injury. However, the highest protection was recorded after 24 h of treatment. Our observations have been supported by an earlier study by Ohta et al. (15) on the preventive effect of melatonin on the progression of alpha-naphthylsocyanate-induced acute liver injury in rats.

Melatonin is five times superior to glutathione in scavenging free hydroxyl radicals. Both methoxy group at position 5 of the indole nucleus and the acetyl group of the side chain of melatonin are essential to scavenge free hydroxyl radicals (10). Melatonin donates an electron to scavenge OH and becomes indolyl cation radical, which in turn neutralises superoxide radical (39).

To verify the decrease in lipid peroxidation manifested by melatonin in benzene-treated rats, we measured GSH. An earlier report from our laboratory showed that circadian rhythms influenced GSH status in the liver of benzene-treated rats (22). GSH levels were lower in rats administered benzene in the morning than in the evening. In the present study melatonin administered in the morning improved the GSH status in the liver of benzene-treated rats. There are reports that pharmacological doses of melatonin given orally to *alpha*-naphthylisothiocyanate- and *N*-acetyl-para-aminophenol (APAP)-treated rats did not affect GSH levels in acute liver injury (15, 16). In contrast, other reports indicate beneficial effects of melatonin on arsenic-induced oxidative stress in the liver and kidney of Wistar rats (18). It was shown recently that expression of genes responsible for oxidative stress and detoxification enzymes is altered by benzene in mice (40). Genes corresponding to the circadian rhythm were also affected by benzene (40). Several reports have confirmed that antioxidative effects of melatonin are caused by at least two different mechanisms, which might, however, be interdependent (41). It has been demonstrated that the glutathione-glutathione peroxidase system suppresses hydroxyl radical generation and prevents oxidative damage and destruction due to this highly reactive radical (42). Our results are supported by these observations.

Our present results show that acute exposure to benzene significantly increased $CYP_{450}2E1$ activity in the liver. Circadian effect of benzene on $CYP_{450}2E1$ has also been demonstrated (22). Although circadian time structure of the $CYP_{450}2E1$ system and of its different isozymes has been well described (43), the effect of melatonin on $CYP_{450}2E1$ after benzene exposure is still unknown. Benzene is chiefly metabolised into phenol, which is excreted in urine. In our earlier study (22), urine phenol in benzene-treated rats was higher in the evening group than in the morning group, showing an inverse relationship with $CYP_{450}2E1$, that was higher in the morning than in the evening group. This suggests that $CYP_{450}2E1$ activity varies with melatonin concentration. Semak et al. (44) believe that microsomal $CYP_{450}2E1$ plays a smaller role in melatonin-O-demethylation in rat liver. Both endogenous and administered melatonin are known to metabolise in humans principally by 6-hydroxylation, with O-demethylation representing a relatively minor pathway. The resulting 6-hydroxymelatonin (6-HMEL) and N-acetyl-5-hydroxytrypamine (N-acetylseratonin) are excreted in urine as their sulphate and glucuronide conjugates (45).

Semak et al. (44) demonstrated that 2-hydroxymelatonin and AFMK were also formed in reactions catalysed by the liver CYP_{450}. They showed that mitochondrial CYP_{450} participated in melatonin metabolism in rat liver. They identified mitochondria as the target of melatonin reactions.

Melatonin increases the activity of the respiratory chain complexes I and IV, inhibits mitochondrial pathways of apoptosis, and participates in the circadian oscillations of oxidative phosphorylation (46, 47). Metabolic pathways of melatonin in microsomes and mitochondria involve the same CYPs. At least in rats, $CYP_{450}2E1$ additionally contributes to melatonin metabolism in the mitochondria. We believe that melatonin expresses antioxidative effects against benzene by accelerating its metabolism through $CYP_{450}2E1$ and GSH concentrations in the liver. Histopathological findings are in agreement with other observations and support this conclusion. This does not undermine the free radical scavenging character of melatonin, but offers an explanation for $CYP_{450}2E1$-mediated protection against benzene toxicity. The absence of prominent lesions in the liver of melatonin-treated rats exposed to benzene only confirms melatonin's antioxidative effects. Our findings may be relevant for occupational health.

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Sažetak

MELATONIN INHIBIRA LIPIDNU PEROKSIDACIJU U JETRI ŠTAKORA UZROKOVANU BENZENOM

Istražena je antioksidacijska uloga melatonina u zaštiti protiv toksičnoga djelovanja benzena u jetri štakora. Utvrđeno je da kratkoročno odnosno dugoročnije liječenje štakora melatoninom u različitoj mjeri štiti štakore istodobno izložene benzenu. Inhibicija lipidne peroksidacije mitohondrija i mikrosoma bila je različita nakon 24 h, 15 dana, odnosno 30 dana liječenja melatoninom. Najveća inhibicija lipidne peroksidacije mitohondrija zamijećena je nakon primjene jednokratne doze melatonina, dok je najizraženija inhibicija u mikrosomima zamijećena nakon 30 dana liječenja melatoninom. Slična istraživanja pokazuju da razina glutationa (GSH) najviše raste nakon 24 h liječenja melatoninom. Nije zamijećena razlika između liječenja u trajanju od 15 odnosno 30 dana. U štakora koji su uz benzen istodobno primali i melatonin razine citokroma P_{450}2E1 pale su nakon 24 h odnosno 15 dana izloženosti. U štakora koji su primali samo melatonin te su razine nakon 30 dana statistički neznačajno porasle u odnosu na skupinu izloženu samo benzenu. Histopatološka analiza jetre načelno je potvrdila ove nalaze. Koncentracije fenola u mokraći bile su niže u štakora koji su istodobno primali melatonin i benzen. Ovi rezultati pokazuju da melatonin utječe na citokrom P_{450}2E1, koji je odgovoran za metabolizam benzena. Inhibira li se njegov metabolizam, smanjuje se lipidna peroksidacija. Zaključak je da melatonin štiti od lipidne peroksidacije uzrokovane benzenom.

KLJUČNE RIJEČI: *CYP_{450}2E1, fenol, GSH, histopatologija, mikrosomi, mitohondriji, mokraća*

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