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Scientific paper

MELATONIN IMPROVES LIVER FUNCTION IN BENZENE-TREATED RATS

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In this study, we investigated the beneficial effects of melatonin against benzene-induced liver function impairments in Wistar rats. After 30 days of treatment, it significantly lowered hepatosomatic indices, bilirubin, and hydroxyproline in male and female benzene-treated rats. Even though it did not influence aspartate aminotransferase, melatonin had beneficial effects on alanine aminotransferase and alkaline phosphatase. Our results suggest that melatonin is an effective modulator of liver function in benzene-treated rats thanks to its antioxidative properties.

KEY WORDS: *alkaline phosphatase, antioxidants, bilirubin, hydroxyproline, serum transaminases*

Benzene is a volatile aromatic hydrocarbon widely used in industry as a solvent. A number of sources such as cigarette smoke, gasoline, and vehicle exhausts contaminate the environment with it (1, 2). Benzene has been evidenced to cause leukaemia and cancer in man and animals (3-5).

Gonasun (6) reported benzene metabolism in mouse liver microsomes. Sawahata and Neal (7) studied the biotransformation of phenol, benzene's major metabolite, to hydroquinone and catechol by rat liver microsomes. Arinç et al. (8) studied the effects of benzene on CYP_{450}-dependent drug-metabolising enzymes (8). In a comprehensive review of various occupational agents, Bacarelli (9) reports about benzene effects on endocrine glands, including the pituitary, in experimental animals and humans. Benzene, toluene, and xylene seem to affect semen quality and the function of accessory glands in exposed workers (10). Benzene induces histopathological lesions to the mouse testis (11). Benzene fumes also affect endocrine activity in rats (12).

It is generally agreed that benzene needs to metabolise to manifest its toxic effects. Oxidative metabolites (epoxides, free radicals, aldehyde, and quinones) of benzene destroy CYP_{450}2E1 (13) and are believed to be responsible for its carcinogenic effects. In an earlier study we investigated the role of oxidative stress in benzene toxicity in rat liver, kidney, and lungs (14).

Melatonin (N-acetyl-methoxytryptamine) functions as a "time giver" (*zeitgeber*) in the regulation of circadian rhythm (15). It is mostly synthesised at night (16). A series of experiments made in our laboratory suggests that hormones change the metabolic disposition of drugs/chemicals (17-21).

However, the effects of melatonin on benzene metabolism and toxicity have not been studied so far. Recently, Reiter (22) described an intriguing antioxidant property of melatonin. An earlier report from our laboratory (23) has shown that melatonin protects against benzene-induced lipid peroxidation in rat liver. This study continues to investigate the protective effects of melatonin, this time against benzene-induced damage of the liver function in rats.

MATERIALS AND METHODS

Sixty male and sixty female three-month-old Wistar rats (150 g±20 g) procured from Jamia Hamdard animal facility in New Delhi were acclimatised to laboratory conditions [room temperature (25±5) °C, humidity (50±10) %, 12-hour dark/light cycle] for 2 weeks. The rats were distributed in four treatment arms, as follows: control – receiving 0.2 mL of 2 % olive oil alone; melatonin – receiving 0.25 mL of 2 % melatonin alone; benzene – receiving 0.2 mL of 2 % benzene alone; and benzene + melatonin - receiving both compounds in the above doses one hour apart. The treatment groups were further divided in three subgroups with ten animals each according to exposure/treatment duration, i.e. 24 h, 15 days, and 30 days.

All animal treatments and protocols had been approved by the Institutional Ethics Committee before we started the experiment.

Treatment

Benzene (AR grade) was procured from S-Merck (Mumbai, India). Predetermined sublethal dose of 0.2 mL of 2 % benzene in olive oil (14) was administered by intramuscular injection at 08:00 a.m. every other day. Melatonin (N-acetyl methoxytryptamine, Sigma Chemical Company, St. Louis, MO, USA) was injected intraperitoneally in the dose of 0.25 mL per 100 g of body weight one hour later (at 9:00 a.m.), as described earlier (23).

Rats were killed under light ether anaesthesia 24 h after the last treatment dose. Over these 24 h, they did not receive any food. Blood was collected from the heart. Serum was separated by centrifugation and processed for liver function tests.

Serum transaminases

Serum transaminases were determined according to the method described by Reitman and Frankel (24). 2,4-dinitrophenyl hydrazine, aspartate aminotransferase (AST) substrate, alanine aminotransferase (ALT) substrate, and pyruvate standard were supplied in the commercial kit procured from Span Diagnostics (Surat, India). Absorbance was recorded at 510 nm using a Systronics visual spectrophotometer (Ahmedabad, India).

Total bilirubin

Serum bilirubin was determined using a commercial kit procured from Ozone Biochemicals Pvt. Ltd. (Gurgaon, India) following the method of Burtis and

Table 1 *Effect of melatonin on the hepatosomatic index (HSI) of benzene treated rats.*

Sex	Treatments	HSI		
		24 h	15 days	30 days
Male	Control	4.96±0.251 (4.23 to 5.93)	4.18±0.232 (3.43 to 4.84)	4.21±0.238 (3.42 to 4.96)
	Melatonin	4.83±0.192* (4.32 to 5.43)	4.19±0.264* (3.22 to 4.69)	4.46±0.183* (3.80 to 5.07)
	Melatonin + benzene	5.87±0.059* (5.68 to 6.01)	4.15±0.260* (3.18 to 4.85)	4.35±0.360* (3.45 to 5.63)
	Benzene	7.10±0.141 N.S. (6.80 to 7.61)	4.37±0.251* (3.35 to 5.01)	4.89±0.357* (3.55 to 5.98)
	F Value	28.53	0.1265	0.7947
Female	Control	4.54±0.329 (3.76 to 5.94)	3.65±0.223 (3.10 to 4.50)	4.09±0.133 (3.75 to 4.63)
	Melatonin	4.19±0.217* (4.05 to 4.93)	4.04±0.129* (3.71 to 4.50)	3.75±0.174* (3.33 to 4.39)
	Melatonin + benzene	5.06±0.181* (4.43 to 5.57)	3.83±0.374* (2.76 to 4.93)	4.41±0.512* (3.73 to 6.69)
	Benzene	6.71±0.144 N.S. (6.10 to 7.05)	3.93±0.217* (3.24 to 4.63)	4.25±0.181 N.S. (3.71 to 4.80)
	F Value	19.77	0.3492	0.7399

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*$p \leq 0.05$*).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Table 2 *Effect of melatonin on the total bilirubin in the serum of benzene treated rats.*

Gender	Treatments	Total bilirubin / mg per 100 mL		
		24 h	15 days	30 days
Male	Control	1.370±0.226 (0.80 to 2.08)	1.370±0.100 (1.02 to 1.75)	1.380±0.228 (0.80 to 2.08)
	Melatonin	0.960±0.124* (0.574 to 1.379)	2.760±0.130 N.S. (2.30 to 3.20)	1.450±0.284* (0.804 to 0.230)
	Melatonin + benzene	1.260±0.107* (0.919 to 1.494)	1.300±0.040* (1.12 to 1.45)	1.940±0.128* (1.60 to 2.31)
	Benzene	1.080±0.140* (0.574 to 1.494)	3.700±0.180 N.S. (3.0 to 4.2)	4.810±0.142 N.S. (4.32 to 5.13)
	F Value	1.145	60.54	62.42
Female	Control	0.680±0.134 (0.344 to 1.149)	1.470±0.072 (1.25 to 1.68)	2.190±0.373 (1.03 to 3.13)
	Melatonin	1.700±0.119 N.S. (1.264 to 2.068)	3.100±0.230 N.S. (2.3 to 3.8)	3.160±0.291 N.S. (2.3 to 3.89)
	Melatonin + benzene	2.750±0.134 N.S. (2.298 to 3.103)	1.410±0.060 N.S. (1.28 to 1.65)	3.830±0.205 N.S. (3.15 to 4.34)
	Benzene	7.050±0.155 N.S. (6.436 to 7.471)	4.080±0.110 N.S. (3.75 to 4.50)	2.460±0.178* (1.91 to 2.85)
	F Value	339.1	65.61	7.234

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*p ≤ *0.05).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Table 3 *Effect of melatonin on the urinary hydroxyproline of benzene treated rats.*

Sex	Treatments	Hydroxyproline / mg L^{-1}		
		24 h	15 days	30 days
Male	Control	4.06±0.172 (3.6 to 4.5)	3.73±0.125 (3.73 to 4.25)	3.82±0.851 (3.43 to 4.20)
	Melatonin	4.15±0.139* (3.7 to 4.5)	4.04±0.213* (3.5 to 4.7)	6.43±0.207 N.S. (5.75 to 6.93)
	Melatonin + benzene	6.40±0.188* (5.92 to 6.83)	5.54±0.209* (4.93 to 6.15)	5.70±0.256* (4.9 to 6.4)
	Benzene	8.14±0.250 N.S. (7.5 to 8.9)	7.44±0.263 N.S. (6.7 to 8.1)	7.09±0.125 N.S. (6.73 to 7.40)
	F Value	104.6	61.76	53.97
Female	Control	3.40±0.209 (2.7 to 3.9)	3.27±0.189 (2.8 to 3.78)	3.39±0.261 (2.83 to 4.2)
	Melatonin	5.02±0.062 N.S. (4.83 to 5.18)	4.23±0.071 N.S. (3.98 to 4.39)	6.18±0.220 N.S. (5.6 to 6.8)
	Melatonin + benzene	4.88±0.196* (4.3 to 5.5)	6.06±0.177* (5.6 to 6.6)	5.40±0.222* (4.85 to 5.98)
	Benzene	6.77±0.144 N.S. (6.23 to 7.03)	6.10±0.251 N.S. (5.5 to 6.9)	6.44±0.191 N.S. (5.83 to 6.93)
	F Value	71.26	57.85	37.65

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*p ≤ *0.05).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Table 4 *Effect of melatonin on the alkaline phosphatase (ALP) activity in the serum of benzene treated rats.*

Sex	Treatments	ALP activity / Karmen unit		
		24 h	15 days	30 days
Male	Control	12.420±3.030 (5.05 to 21.0)	12.440±0.190 (11.8 to 13.0)	12.41±0.266 (11.7 to 13.2)
	Melatonin	28.570±0.518 N.S. (27.03 to 30.29)	17.800±0.250 N.S. (17 to 18.6)	14.03±0.021 N.S. (13.91 to 14.98)
	Melatonin + benzene	26.110±0.371* (24.77 to 26.94)	20.180±0.290 * (19.3 to 21.3)	18.60±0.153* (13.9 to 23.1)
	Benzene	22.360±0.260 N.S. (21.55 to 23.29)	27.700±0.059 N.S. (26.3 to 30.3)	19.90±0.107 N.S. (17.0 to 23.0)
	F Value	20.65	217.3	13.77
Female	Control	20.800±0.455 (19.59 to 22.33)	15.040±0.210 (14.3 to 15.8)	12.52±0.234 (11.98 to 13.31)
	Melatonin	9.160 ±0.347 N.S. (18.12 to 10.29)	13.160±0.170 N.S. (12.7 to 13.9)	14.12±0.184 N.S. (13.71 to 14.63)
	Melatonin + benzene	19.330±0.374* (18.25 to 20.46)	17.100±0.180* (16.6 to 17.8)	17.12±0.203* (16.5 to 17.6)
	Benzene	15.710±0.643 N.S. (13.81 to 17.68)	20.100±0.390 N.S. (18.6 to 21.3)	19.70±0.097 N.S. (13.5 to 19.37)
	F Value	98.11	97.08	155

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*$p \leq 0.05$*).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Table 5 *Effect of melatonin on the aspartate transaminase (AST) activity in the serum of benzene treated rats.*

Sex	Treatments	AST activity / Karmen unit		
		24 h	15 days	30 days
Male	Control	42.40±4.400 (30.0 to 52.0)	42.80±1.590 (38.0 to 48.0)	41.80±3.786 (31.0 to 47.0)
	Melatonin	275.6±1.460 N.S. (271.0 to 280.0)	50.80±1.760 N.S. (46.0 to 58.0)	45.40±3.075* (37.0 to 53.0)
	Melatonin + benzene	340.2±1.030 N.S. (337.0 to 344.0)	31.40±1.640* (27.0 to 38.0)	58.00±4.816* (41.0 to 69.0)
	Benzene	330.6±1.220 N.S. (327.0 to 335.0)	36.40±2.000 N.S. (30.0 to 43.0)	68.20±3.891 N.S. (57.0 to 79.0
	F Value	3085	16.74	9.414
Female	Control	120.8±5.240 (122.0 to 198.0)	76.00±1.950 (70.0 to 82.0)	72.20±3.967 (63.0 to 86.0)
	Melatonin	239.6±1.280 N.S. (235.0 to 243.0)	40.20±1.590 N.S. (35.0 to 46.0)	62.20±2.634 N.S. (59.0 to 71.0)
	Melatonin + benzene	265.4±1.460* (261.0 to 270.0)	45.00±1.466* (40.0 to 50.0)	44.80±2.437* (37.0 to 51.0)
	Benzene	142.8±1.660 N.S. (137.0 to 148.0)	42.20±1.840 N.S. (37.0 to 49.0)	72.20±2.817 N.S. (65.0 to 81.0)
	F Value	78.29	70.46	18.27

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*$p \leq 0.05$*).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Table 6 *Effect of melatonin on the alanine transaminase (ALT) activity in the serum of benzene treated rats.*

Sex	Treatments	ALT activity / Karmen unit		
		24 h	15 days	30 days
Male	Control	71.80±2.033 (65.0 to 78.0)	71.40±1.749 (67.0 to 76.0)	71.00±3.741 (61.0 to 80.0)
	Melatonin	67.60±1.715* (62.0 to 73.0)	140.0±2.830 N.S. (130.0 to 148.0)	105.6±6.185 N.S. (88.0 to 120.0)
	Melatonin + benzene	61.60±2.816* (50.0 to 68.0)	124.4±1.890* (118.0 to 130.0)	82.80±5.739* (87.0 to 103.0)
	Benzene	66.00±1.360 N.S. (62.0 to 70.0)	127.2±2.210 N.S. (121.0 to 135.0)	83.80±7.598* (65.0 to 108.0)
	F Value	3.391	144.60	5.961
Female	Control	62.60±1.594 (58.0 to 68.0)	84.20±1.960 (78.0 to 90.0)	94.80±7.480 (75.0 to 115.0)
	Melatonin	73.20±1.537 N.S. (69.0 to 78.0)	105.2±2.070 N.S. (98.0 to 112.0)	110.0±4.959* (95.0 to 125.0)
	Melatonin + benzene	71.00±0.896* (68.0 to 74.0)	138.6±1.940* (132.0 to 145.0)	84.20±7.358* (63.0 to 103.0)
	Benzene	68.20±1.280 N.S. (64.0 to 72.0)	143.8±2.020 N.S. (138.0 to 150.0)	87.80±5.686* (72.0 to 105.0)
	F Value	9.189	150.80	3.119

*Results are expressed as mean±S.E. (*n=*5)*
** denotes values significantly different from control (*$p \leq 0.05$*).*
N.S. denotes non significant differences from control.
Values in parenthesis indicate the range.

Ashwood (25). Absorbance was recorded at 540 nm using a Systronics visual spectrophotometer (Ahmedabad, India).

Alkaline phosphatase

Serum alkaline phosphatase (ALP) was determined using a commercial kit procured from Span Diagnostics (Surat, India), following the method described by Kind and King (26).

Hydroxyproline

Hydroxyproline was estimated in urine samples using the colorimetric method of Pondenphant et al. (27). Absorbance was recorded at 550 nm using a Systronics visual spectrophotometer (Ahmedabad, India).

Statistical analysis

Student's *t*-test was used to compare differences between the experimental groups. The level of significance between means was set at $p<0.05$. All calculations were performed using the IBM SPSS software (IBM, New Delhi, India). Inter-group comparisons were made using one-way analysis of variance (ANOVA).

RESULTS

Benzene treatment increased the hepatosomatic index (liver to body weight ratio) of both male and female rats, but was higher in male rats. However, melatonin seems to have countered its effects in the benzene + melatonin arm (Table 1). Similar was found for bilirubin (Table 2) and hydroxyproline (Table 3).

Benzene also increased ALP, more so in male than in female rats. Melatonin countered the effects of benzene only after 15 and 30 days of treatment (Table 4).

AST significantly increased after 24 h of benzene treatment. Its activity dropped after 15 days, but increased again after 30 days. Melatonin showed no significant effect on AST activity (Table 5).

At hour 24, benzene lowered ALT activities in male, but increased them in female rats. Enzyme activities increased after 15 days of treatment and then

dropped on treatment day 30, still remaining above control activities. Melatonin following benzene treatment did not significantly lower enzyme levels in either male or female rats, regardless of treatment duration (Table 6).

DISCUSSION

An earlier report from our laboratory has shown that benzene is a potent hepatotoxin (28). Our gene expression study (29) also confirmed that downregulation of CYP2610 and CYP4A10 genes after benzene treatment accounts for biochemical and histopathological changes in BALB/c mice. We also observed that circadian rhythm affects benzene-induced lipid peroxidation (30). Another benzene-induced lipid peroxidation modulator in rat liver that we found was melatonin (23). This urged us to find out how much in fact melatonin protects the liver in benzene-treated rats. Although the effects of a few other hormones have been described earlier (31), this is the first report on such effects by melatonin. The action of melatonin, however, is not restricted to neuroendocrine physiology. Since 1993, melatonin has been known to scavenge ROS, including singlet oxygen (O_2), superoxide anion radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and lipid peroxide radical ($LOO^{\bullet}$) (32-35). It also acts as an indirect antioxidant through the activation of major antioxidant enzymes including SOD, CAT, and GPx (36-37). Improved liver function after melatonin treatment seems to be a consequence of this effect.

In the first 24 h, melatonin could not stop transaminases to leak into the bloodstream of benzene-treated rats. This can be explained by the enormous functional reserves of the liver parenchyma. Serum enzyme activities regress as the injury progresses, which suggests that substantial serum AST or ALT does not necessarily reflect cell death. AST is found in a wide variety of tissues beside liver, including heart, skeletal muscles, kidney, and brain, whereas ALT appears to be primarily located in the liver (38).

Our alkaline phosphatase findings seem to confirm the protective effects of melatonin against benzene-induced hepatotoxicity. Several physiological factors have been reported to affect serum AP activity including age, sex, and gestation (39). Elevations in serum AP are associated with a wide variety of lesions beside the liver. In other words, benzene could have raised serum AP levels through both hepatic and non-hepatic disorders (40). Melatonin may have acted through several physiological effects on serum alkaline phosphatase activity, such as membrane stabilisation and reduced electron leakage (41).

All groups that received benzene had higher serum bilirubin. Bilirubin is mainly derived from the haem moiety of haemoglobin, liberated when the affected red cells are removed from circulation by the reticuloendothelial system (42). Hyperbilirubinaemia is not considered sensitive enough for early detection of parenchymal liver disease (43), which explains why 24-hour exposure to benzene did not result in hyperbilirubinaemia. However, 15 and 30-day exposure did. In the benzene + melatonin groups, melatonin seems to have exerted its protective effects, probably through glucuronidation of benzene metabolites (phenol, catechol, and hydroquinone) (44). Phase-I biotransformation products are deactivated by phase-II biotransformation reactions, including glucuronidation, sulphonation, acetylation, methylation, and the formation of mercapturic acid. Glucuronidation may have increased the hydrophilicity of phenol/hydroquinone/catechol, promoting their excretion. Since phase-II enzymes are located mainly in the cytosol, these reactions occur faster than phase-I reactions. Melatonin might have contributed to detoxification by stimulating responsible phase-II enzymes.

Hydroxyproline is a reliable marker of collagen metabolism. Morphologic and biochemical alterations in collagen occur under diverse pathological conditions (45). However, the effects of benzene on collagen have not yet been studied. Our results show that benzene disturbs collagen metabolism. Melatonin treatment, in turn, inhibits collagenolysis and thus restores hydroxyproline to normal levels.

Our findings suggest that melatonin can be used to buffer benzene toxicity in solvent industry workers. Reiter et al. (46) have discussed the pathophysiological implications of melatonin against free radical-mediated disorders. Effects of melatonin as an antioxidant include (i) direct free radical scavenging; (ii) stimulation of antioxidant enzymes; and (iii) boosting the efficiency of mitochondrial oxidative phosphorylation and other antioxidants. Other laboratories have conducted similar experiments with other antioxidants such as quercetin (47). Since benzene-induced hepatotoxicity primarily involves oxidative stress (48), melatonin owes a large part of its protective effect to its antioxidative properties.

Further studies of antioxidant enzymes are needed to support of this conclusion.

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Sažetak

MELATONIN MIJENJA JETRENU FUNKCIJU U ŠTAKORA IZLOŽENIH BENZENU

Svrha je ovog ispitivanja bila utvrditi zaštitno djelovanje melatonina od oštećenja jetrene funkcije izazvanog benzenom u Wistar štakora. Nakon 30-dnevne primjene, melatonin je značajno snizio hepatosomatski indeks te razine bilirubina i hidroksiprolina u muških i ženskih štakora. Premda nije utjecao na aspartat aminotransferazu, povoljno je djelovao na alanin aminotransferazu i alkalnu fosfatazu. Naši rezultati upućuju na to da melatonin djelotvorno mijenja jetrenu funkciju u štakora izloženih benzenu, upravo zbog svojih antikoksidativnih svojstva.

KLJUČNE RIJEČI: *alkalna fosfataza, antioksidansi, bilirubin, hidroksiprolin, serumske transaminaze*

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