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# Melatonin protects against renal oxidative stress after obstructive jaundice in rats

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**Abstract**

The goals of this study were to analyze the renal oxidative status in experimental biliary obstruction and to evaluate the impact of melatonin on renal oxidative stress. Cholestasis was done by double ligature and section of the extra-hepatic biliary duct. Melatonin was injected i.p. (500 μg/kg/day). Malondialdehyde, reduced glutathione, catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase and glutathione transferase were determined in the renal tissue. After biliary obstruction, an increase in malondialdehyde ($P < 0.0001$) and a fall in reduced glutathione ($P < 0.0001$) were seen. Moreover, the scavenger enzyme activity had significantly diminished. After melatonin administration, the malondialdehyde fell significantly ($P < 0.0001$), whereas reduced glutathione showed an important increase ($P < 0.0001$) compared with the ligated bile duct group. Experimental bile duct obstruction was associated to an increase of renal oxidative stress. Treatment with melatonin decreased the renal lipid peroxidation, and both the reduced glutathione as well as the scavenger enzyme activity recovered. © 2001 Published by Elsevier Science B.V.

*Keywords:* Biliary obstruction; Renal oxidative stress; Melatonin

## 1. Introduction

Renal failure is an important postoperative complication associated to obstructive jaundice. The mean incidence of acute renal failure in different series is approximately 8% (Fogarty et al., 1995). Since the association between obstructive jaundice and renal dysfunction was reported (Clairmont and von Haberer, 1910), several hypotheses about the pathogenesis of renal failure in biliary obstruction have been proposed, including nephrotoxicity of hyperbilirubinaemia and bile salt (Armtrong et al., 1984; Aoyagi and Lowenstein, 1968), endotoxaemia (Bailey, 1976), alterations in the systemic and renal haemodynamic systems (Bomzon and Kew, 1983), fluid depletion (Gallardo et al., 1998; Padillo et al., 1999) and oxidative stress (Bomzon et al., 1997). Oxidative stress can also play an essential role on the renal dysfunction associated to obstructive jaundice through the formation of several vasoactive mediators, as well as by direct effect on glomerular microcirculation by causing the contraction of mesangial cells, and thus decreasing the glomerular capillary ultrafiltration coefficient (Bomzon et al., 1997). On the other hand, the main hormone of the pineal gland, melatonin, is a powerful antioxidant that has demostrated the capacity of reducing lipid peroxidation (Montilla López et al., 2000), and increasing the antioxidant enzymes activity in experimental models of biliary obstruction (Montilla et al., 2001). Thus, melatonin might improve the kidney oxidative stress injury markers, as well as the scavenger antioxidant enzymes after bile duct obstruction. Experimental study was designed to confirm this hypothesis.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats, weighing between 250 and 330 g at the beginning of the study, were purchased from Charles River of Barcelona, Spain. They were subjected to controlled conditions of temperature (about 22 °C), illumination (12 h light:12 h dark cycle) and provided with food (Purina®, Barcelona, Spain) and water ad libitum.

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Table 1
Results of bilirubin direct and oxidative stress parameters in sham operated group ($n = 6$) and in animals with bile duct ligation ($n = 6$)

| Parameters | Sham operated | Ligated bile duct |
|---|---|---|
| Direct bilirubin (mg/dl) | 0.07 | $6.68 \pm 0.42$[a] |
| Oxidative stress (μmol/g tissue) | | |
| Malondialdehyde | $8.05 \pm 0.25$ | $11.01 \pm 0.07$[a] |
| Reduced glutathione | $6.29 \pm 0.16$ | $2.05 \pm 0.07$[a] |
| Scavenger enzymes (activity unit/g tissue) | | |
| Catalase (×1000) | $10.41 \pm 0.41$ | $7 \pm 0.57$[b] |
| Superoxide dismutase | $5.90 \pm 0.07$ | $4.55 \pm 0.13$[a] |
| Glutathione reductase | $2.40 \pm 0.01$ | $1.71 \pm 0.02$[a] |
| Glutathione peroxidase | $4.38 \pm 0.06$ | $1.97 \pm 0.09$[a] |
| Glutathione transferase | $4.53 \pm 0.06$ | $3.18 \pm 0.10$[a] |

[a] $P < 0.0001$.
[b] $P < 0.001$.

### 2.2. Experimental design

Six animals were included in each of the following three groups: (1) sham operated: dissection of the bile duct; (2) obstructive jaundice: double ligature and section of the extra-hepatic bile duct; and (3) obstructive jaundice plus melatonin.

All the surgical procedures were done with the animals under anesthesia with ketamine (4 mg/kg/i.p.) and midazolam (80 mg/kg/i.p.). Cephazoline (17 mg/kg/i.m.) was used as antibiotic prophylaxis. After a midline incision under sterile conditions, the common bile duct was exposed. A double ligature with silk suture was done and the bile duct was sectioned. A two-layer running suture was used for abdominal closure with Dexon® (Braun Dexon Barcelona, Spain) and Mersilk® (Ethicon, Brussels, Belgium).

Crystalline melatonin was purchased from Sigma (St. Louis, MO, USA). The solvent used for melatonin was a mixture of ethanol (5%) and NaCl (0.9%). The injected dose of melatonin was 500 μg/kg/day/i.p. Melatonin was injected daily at 10 h, beginning 1 day before the ligature and for 7 days running.

### 2.3. Biochemical parameters

The animals were sacrificed under the same anesthesic conditions, 24 h after the last injection. Blood samples were collected by exsanguination through an abdominal aorta puncture and put into chilled heparinized tubes, which were centrifuged at 3000 rpm for 10 min at 4 °C. Thereafter, the kidneys were removed and cut into pieces individually separated, before preparing homogenates which were frozen −20 °C for later study.

Direct bilirubin was measured in serum using an Axon autoanalyzer (Bayer Diagnostics, Tarrytown, NY, USA).

The contents of lipid peroxides, measured as malondialdehyde, and reduced glutathione were determined in kidney homogenates, using reagents purchased from Bioxytech (Portland, USA), LPO-585 and reduced glutathione-400, respectively. Units used for these determinations were μmol/g of tissue.

The scavenger enzymes activity, glutathione peroxidase and glutathione reductase were measured according to the Flohé and Gunzler (1984) method. Glutathione transferase activity was determined according to the technique described by Warholm et al. (1985). Enzymes activity catalase and superoxide dismutase were measured according to Aebi (1984) and Sun et al. (1988), respectively. All enzymes were determined in kidney tissue as activity units per gram of tissue.

### 2.4. Statistical analysis

A two-tailed, nonpaired, Student's *t*-test was used to evaluate differences between means. Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Baseline profile

As expected, after a proper ligature of the main bile duct, an increase in serum bilirubin was observed (Table 1). Moreover, the acute biliary obstruction determined an oxidative stress status in the kidney, characterized by a significant increase of malondialdehyde in the renal tissue and a significant fall in reduced glutathione obtained in the kidney (Table 1).

The activity of all scavenger enzymes measured in this study (catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase and glutathione transferase) was significantly lower in the group with biliary obstruction than in the sham operated group (Table 1; Figs. 1 and 2.

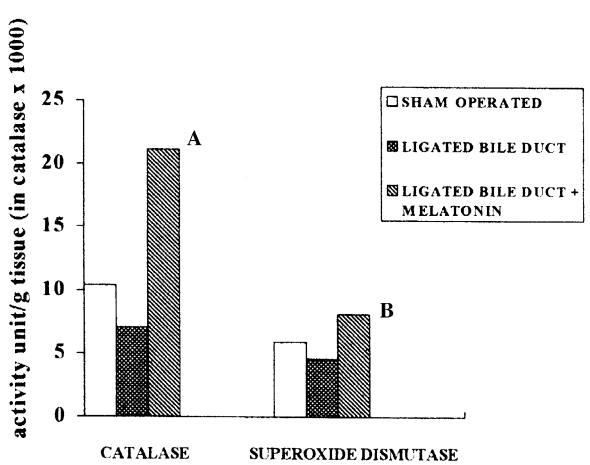


Fig. 1. Effect of melatonin on renal catalase and superoxide dismutase activity. (A) $P < 0.0001$ and (B) $P < 0.001$ with regard to sham operated.

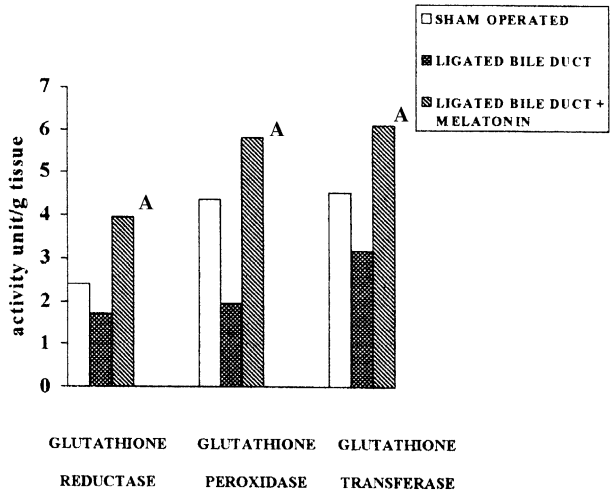


Fig. 2. Impact of melatonin on enzymes associated to glutathione activity. (A) $P < 0.0001$ with regard to sham operated.

### 3.2. Effects of melatonin administration

Direct bilirubin decreased significantly in the ligated bile duct and in the melatonin group compared to the control group (Table 2). Regarding oxidative stress parameters, when the rats were protected with melatonin both malondialdehyde and reduced glutathione, returned to normal values (Table 2). Malondialdehyde levels in the jaundiced rats treated with melatonin were even lower than in the sham operated group ($7.04 \pm 0.24$ vs. $8.05 \pm 0.25$ μmol/g tissue; $P < 0.04$). Similarly, after melatonin administration, the renal reduced glutathione concentration increased in the cholestatic animals to values higher than in the sham operated group ($9.21 \pm 0.15$ vs. $6.29 \pm 0.16$ μmol/g tissue; $P < 0.0001$).

Melatonin also showed an important effect on the antioxidant enzymatic system. In fact, rats protected with the antioxidant drug had an increase in the activity of all scavenger enzymes that were studied in this report. As observed with the reduced glutathione, the increase in the activity of scavenger enzymes after the melatonin administration was higher than in the sham operated group (Table 3; Figs. 1 and 2.

Table 2
Comparison of serum bilirubin and oxidative stress markers in rats with ($n = 6$) and without ($n = 6$) melatonin treatment after bile duct obstruction

| Parameters | Ligated bile duct | Ligated bile duct + melatonin |
|---|---|---|
| Direct bilirubin (mg/dl) | $6.68 \pm 0.42$ | $3.54 \pm 0.11$[a] |
| Oxidative stress (μmol/g tissue) | | |
| Malondialdehyde | $11.01 \pm 0.07$ | $7.04 \pm 0.24$[a] |
| Reduced glutathione | $2.05 \pm 0.07$ | $9.21 \pm 0.15$[a] |
| Scavenger enzymes (activity unit/g tissue) | | |
| Catalase (×1000) | $7 \pm 0.57$ | $21.16 \pm 1.49$[a] |
| Superoxide dismutase | $4.55 \pm 0.13$ | $8.09 \pm 0.32$[a] |
| Glutathione reductase | $1.71 \pm 0.02$ | $3.95 \pm 0.05$[a] |
| Glutathione peroxidase | $1.97 \pm 0.09$ | $5.82 \pm 0.13$[a] |
| Glutathione transferase | $3.18 \pm 0.10$ | $6.11 \pm 0.15$[a] |

[a] $P < 0.0001$.

Table 3
Scavenger enzymes in jaundiced rats treated with melatonin compared with sham operated group

| Scavengers enzymes (activity unit/g tissue) | Sham operated | Ligated bile duct + melatonin |
|---|---|---|
| Catalase (×1000) | $10.41 \pm 0.41$ | $21.16 \pm 1.49$[a] |
| Superoxide dismutase | $5.90 \pm 0.07$ | $8.09 \pm 0.32$[b] |
| Glutathione reductase | $2.40 \pm 0.01$ | $3.95 \pm 0.05$[a] |
| Glutathione peroxidase | $4.38 \pm 0.06$ | $5.82 \pm 0.13$[a] |
| Glutathione transferase | $4.53 \pm 0.06$ | $6.11 \pm 0.15$[a] |

[a] $P < 0.0001$.
[b] $P < 0.001$.

## 4. Discussion

Our study showed that melatonin plays an important role as a protective factor of renal oxidative injury secondary to experimental bile duct ligation. Although obstructive jaundice is a systemic disease where several important organs can be damaged, we focused our study to know the oxidative renal injury in the biliary obstruction. Malondialdehyde is an end product of peroxidative decomposition of polyenic fatty acids in the lipid peroxidation process. The malondialdehyde accumulation in tissues is indicative of the extent of lipid peroxidation (Halliwell, 1991) and oxidative stress. Recently, it has been demostrated (Ljubuncic et al., 2000) that not all plasma malondialdehyde come from the liver and the biliary tree. As previously observed (Ljubuncic et al., 2000; Tajiri et al., 1995), this work showed that experimental cholestasis is associated to a significant increase of renal malondialdehyde regarding the control group. Oxidative stress in renal tissue after bile obstruction may be linked to chronic exposure of tissues to elevated plasma concentration of catecholamines, angiotensin (Laursen et al., 1997) and bile acids (Ljubuncic et al., 2000). Bile acids are accumulated in the liver and plasma of patients with cholestatic liver disease (Ostrow, 1993) and are pro-oxidant, causing direct tissue damage mediated by free radicals (Sokol et al., 1995), or indirectly through the activation of tissue resident macrophages to release free radicals (Ljubuncic et al., 1996). Furthemore, bile acids can also accumulate intracellularly by virtue of their ability to bind to low density lipoprotein (Ceryak et al., 1993), and thus to generate free radicals into the intracellular milieu of extrahepatic tissues due to bile acid spillover into systemic circulation, when the integrity of the enterohepatic circulation is disrupted (Ljubuncic et al., 2000). The reduced glutathione is an

important cellular antioxidant because of its elevated intracellular concentration and also for being the substratum of essential scavenger enzymes for the maintenance of oxidative balance. In this study, a significant fall in the renal reduced glutathione amount was found, suggesting an important consumption against the oxidative injury. Moreover, this decrease of reduced glutathione might suggest that the kidney is unable to recycle reduced glutathione from oxidized glutathione by the glutathione reductase enzyme. There is controversy about the behavior of renal reduced glutathione in the status of oxidative stress in biliary obstruction. In a similar model, a decrease of renal reduced glutathione was reported (Gonzalez-Correa et al., 1997). On the other hand, a significant increase for reduced glutathione in animals with cholestasis vs. control rats was demostrated, arguing that in obstructive jaundice there is a block in the reduced glutathione transport towards the biliary tree, and a large part of the hepatic reduced glutathione is excreted into the systemic circulation and reaches the kidney (Tajiri et al., 1995). Recent studies, however, have demostrated a significant fall both in hepatic and plasma reduced glutathione in rats with biliary obstruction (Montilla et al., 2001). The renal antioxidant enzymatic system has also been injured by oxidative stress in this disease. In the present study, we have evaluated the activity of five enzymes, three related to reduced glutathione (glutathione reductase, glutathione peroxidase and glutathione transferase), apart from the superoxide dismutase and catalase. All their activities play an essential role of cellular defense against free radicals. We demostrated a decrease in the activity of all enzymes studied deteriorating the oxidative renal imbalance. Other authors have found a significant fall in renal glutathione peroxidase (Gonzalez-Correa et al., 1997) and no changes in superoxide dismutase and glutathione peroxidase (Orellana et al., 2000).

In an effort to control the renal oxidative injury, melatonin was administered to the animals. The melatonin is the main hormone of the pineal gland and its antioxidant properties were discovered in 1993 (Reiter et al., 1993). Our research group has demostrated an improvement not only in oxidative stress parameters measured in plasma and liver, but also in the hepatic scavenger enzymes (Montilla López et al., 2000; Montilla et al., 2001). Furthermore, we previously demostrated the beneficial effects of melatonin in models of nephropathy induced by adriamicyn (Montilla et al., 1997) and diabetes induced by streptozotozin (Montilla et al., 1998). Melatonin protects DNA molecules, proteins, and biologicals membrane lipids from the deleterious effects of free radicals, as for example, the highly toxic hydroxyl radical through electron donation with the generation of indolyl cation radical, a molecule also able to scavenge the superoxide radical (Reiter et al., 1997). This work demostrated that melatonin can carry out a significant lipid peroxidation reduction measured by a decrease in renal malondialdehyde. After melatonin administration, the renal reduced glutathione had increased significantly in the group with cholestasis, compared to both the ligated bile duct and sham operated groups. This increase can be partially explained by an activation of glutathione reductase enzyme, as seen below. Besides this form to improve oxidative damage, melatonin has other mechanisms by which it reduces oxidative injury. Some free radicals can be metabolized by enzymes that are part of the antioxidant network (Reiter et al., 1999). Several of these enzymes have been shown to be stimulated by melatonin. Thus, melatonin has been found to promote the glutathione peroxidase (Barlow-Wladen et al., 1995) and the glutathione reductase activity (Pablos et al., 1998). Later, we found that melatonin increased the activity of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione transferase measured in the liver in the experimental biliary obstruction (Montilla et al., 2001).

In summary, the renal oxidative stress status observed in experimental obstructive jaundice was significantly ameliorated by melatonin administration.

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