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Effects of Intraperitoneal Melatonin on Caustic Sclerosing Cholangitis Due to Scolicidal Solution in a Rat Model

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ABSTRACT

BACKGROUND: Hydatid disease is a worldwide health problem. Treatment is surgical or percutaneous, using scolicidal agents. Caustic sclerosing cholangitis might develop after the contact of scolicidal agents with the biliary ducts. Melatonin, an antioxidant, anti-inflammatory, and anticarcinogenic agent, might be used in the treatment of caustic sclerosing cholangitis due to its possible preventive effects on fibrosis and cell damage.

OBJECTIVE: The aim of the study was to investigate the effects of melatonin on an experimentally developed caustic sclerosing cholangitis with scolicidal solution (formalin) in a rat model.

METHODS: Forty female Sprague-Dawley rats aged 11 to 13 weeks and weighing 250 ± 30 g were randomly assigned to 1 of 4 groups of 10: formalin 5% at 0.5 mL/d + melatonin placebo; formalin placebo + intraperitoneal melatonin 10 mg/kg/d; formalin 5% at 0.5 mL/d + melatonin 10 mg/kg/d; and formalin placebo and melatonin placebo (control). Hepatobiliary function was assessed using dynamic scintigraphy with technetium-99m–mebrofenin on study day 60. The histology of the liver and biliary duct specimens was examined on study day 60. In each group, histopathologic alterations were scored as absent, slight, mild, or severe.

RESULTS: Mean severity scores for parenchymal necrosis in the liver ($P < 0.01$), portal fibrosis ($P < 0.01$), biliary duct proliferation ($P < 0.001$), cholangitis/pericholangitis ($P < 0.01$), hyperemia in the biliary ducts ($P < 0.01$), and fibrosis ($P < 0.01$) were significantly lower in rats treated with formalin + melatonin compared with those treated with formalin alone. No significant differences were observed between the 3 treatment groups with respect to $t_{1/2}$, a parameter used to assess the secretion function of the hepatocytes. However, the $t_{1/2}$ was significantly longer in the treatment groups compared with controls ($P < 0.001$).

CONCLUSION: In this experimental study in a rat model of caustic sclerosing cholangitis, the histopathologic and scintigraphic findings suggested that melatonin is effec-

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tive in attenuating the damage caused by scolicidal agents on the liver and biliary ducts. (*Curr Ther Res Clin Exp*. 2010;71:118–128) © 2010 Excerpta Medica Inc.

KEY WORDS: melatonin, hydatid cyst, scolicidal solutions, caustic sclerosing cholangitis.

INTRODUCTION

Hydatid disease, also known as *echinococcosis,*[1] is a parasitic infestation characterized by cysts on the liver, lungs, and/or kidneys. Hydatid disease affects people living in or visiting endemic regions, especially Mediterranean, Middle Eastern, and African countries, as well as India and Australia.[2] Hydatid disease is a public health issue with serious economic implications in affected countries.[1] The most common site for a hydatid cyst is the liver, and in 5% to 10% of cases, the biliary ducts are also affected.[3] In cases in which the biliary ducts are affected, mortality and morbidity are increased, and treatment is affected.[2]

Treatment of hepatic hydatid cysts is surgical or percutaneous. During surgery, scolicidal agents (hypertonic saline 20%, povidone iodine 1%, silver nitrate 0.5%, or 5% formalin) are injected into the cyst to deactivate the scolices. However, experimental and clinical studies have reported that hepatic and biliary duct damage and caustic sclerosing cholangitis[4–13] may result from contact between these agents and the liver and/or biliary duct during treatment.[3] Sclerosing cholangitis is a chronic cholestatic disease characterized by inflammation, fibrosis, and stenosis of the intrahepatic and extrahepatic biliary ducts. During the natural progression of the disease, progressive biliary duct obstruction, biliary cirrhosis, hepatic failure, and cholangiocarcinoma might be observed.[14]

Synthesized in the pineal gland from tryptophan, melatonin is an endogenous antioxidant agent, the effects of which are mediated through specific receptors found primarily in the brain and peripheral tissues. Melatonin has reported immunestimulating, anti-inflammatory, and anticarcinogenic effects; protective effects on cells, tissues, and organs against oxidative damage[3,15–17]; and preventive effects on fibrosis.[3,15] As such, melatonin might be useful for attenuating caustic sclerosing cholangitis after surgical treatment of hepatic hydatid cysts.

In an experimental study, Montilla et al[18] assessed the effects of melatonin and vitamin E on the cholestasis syndrome, and the preventive effects on hepatic damage, after extrahepatic biliary duct ligation in rats. A secondary objective was to investigate the activity of antioxidant enzymes after treatment with these antioxidant drugs. They reported that melatonin was associated with a significantly hepatoprotective effect, including reductions in the negative parameters of cholestasis (serum bilirubin, $P < 0.05$; alkaline phosphatase, $P < 0.01$; and γ-glutamyl-transpeptidase, $P < 0.001$) and the degree of oxidative stress (malondialdehyde, $P < 0.001$; glutathione, $P < 0.001$) compared with rats with extrahepatic biliary ligation. In a rat model of obstructive jaundice, Padillo et al[19] found that melatonin was associated with protective effects against apoptosis and necrosis in hepatic damage. The authors investigated hepatic damage with the measurement of alanine aminotransferase and direct biliru-

bin. Detection of apoptosis was assessed in fixed liver sections using immunohistochemistry with double-stranded breaks that occurred during apoptosis. The sections were labeled with biotinylated nucleotides by terminal deoxynucleotidyl-transferase. The authors reported that the administration of melatonin was associated with significant reductions in DNA fragmentation induced by cholestasis (*P* not reported) and that the obstruction of extrahepatic biliary duct was associated with significantly increased concentrations of direct bilirubin ($P < 0.001$) and aminotransferase ($P < 0.01$). However, a literature search found no reports of the effects of melatonin on sclerosing cholangitis.

This study investigated the effects of melatonin, administered with a scolicidal solution (formalin), on the attenuation of damage of the liver and biliary ducts in a rat model of caustic sclerosing cholangitis.

MATERIALS AND METHODS

Study Design

The study was conducted at the Laboratory Animals Care Unit in accordance with the guidelines for the care and use of laboratory animals established by the Animal Ethics Committee, which approved the study design. Forty female Sprague-Dawley rats aged 11 to 13 weeks and weighing 250 ± 30 g were studied. Using a computer-generated table of random numbers, rats were assigned to 1 of 4 groups of 10: formalin (lot no. F-22432, Eczacibasi Ilac Sanayi, Levent, Istanbul, Turkey) 5% at 0.5 mL/d + melatonin placebo (formalin group); intraperitoneal melatonin (lot no. M-5250, Sigma Chemical Co., Steinheim, Germany) 10 mg/kg/d + formalin placebo (melatonin group); formalin 5% at 0.5 mL/d + melatonin 10 mg/kg/d (formalin + melatonin group); and formalin placebo + melatonin placebo (control group).

The animal room was maintained at a temperature of 22°C ± 2°C and a relative humidity of 55% ± 15%, with a 12-hour light–dark cycle. Tap water was freely available throughout the acclimatization and study periods. The schedule of the experiment was constructed as formalin administration on study day 1, melatonin administration on days 1 to 14, and hepatobiliary scintigraphy and euthanasia on day 60.

Formalin Administration

Each rat was anesthetized using 5 mg/kg of xylazine and 30 mg/kg of ketamine hydrochloride intramuscularly before surgery. After laparotomy, biliary ducts of the rats administered formalin or formalin + melatonin were cannulized using a 33-G catheter, and 0.5 mL of 5% formalin was administered. The biliary duct was clamped at the distal end for ~5 minutes. The same procedure was used in the melatonin and control groups, and 0.5 mL of 0.09% NaCl was administered through the biliary duct.

Melatonin Administration

Rats in the melatonin and formalin + melatonin groups were administered 10 mg/kg/d of melatonin intraperitoneally for 14 days, starting on study day 1. The formalin and control groups received the same amount of 0.09% NaCl intraperitoneally for 14 days.

Hepatobiliary Scintigraphy

On day 60, before euthanasia, hepatobiliary activity was detected in all 4 animal groups using a 37-MBq injection of technetium-99m (^{99m}Tc)–mebrofenin (Bridatech, Amersham International, London, United Kingdom), a bilirubin analogue pharmaceutically used in hepatic scintigraphy, via the tail vein. The scintigrams were examined by a blinded nuclear medicine specialist. Dynamic scintigraphy, a noninvasive and repeatable method used to assess the liver and biliary ducts, was conducted for 60 minutes (1 frame/min). Following intravenous injection of mebrofenin, almost all of the mebrofenin is taken up rapidly by hepatocytes and secreted into the biliary ducts.

On the dynamic images, circular regions of interest (ROIs) were drawn on the upper right quadrant of the liver to exclude the activities of the major biliary tract, gut, and right kidney. From these ROIs, time–activity curves were generated for each group, and time of peak uptake (TPU) and $t_{1/2}$ were calculated.

TPU is used to assess the extraction function of the hepatocytes, and ^{99m}Tc-mebrofenin denotes the time from injection to highest concentration of mebrofenin in the liver. A higher ^{99m}Tc-mebrofenin value is a quantitative indicator of impaired hepatocyte extraction function. The $t_{1/2}$ value was used for the assessment of the secretion function of the hepatocytes because it indicates the rate of extraction of ^{99m}Tc-mebrofenin, concentrated by hepatocytes after extraction, into the biliary duct. The $t_{1/2}$ of peak activity was used for the measurement of the secretion rate, and a delay in this time suggests impaired secretory function of the hepatocytes.

Histopathologic Assessment of Liver and Biliary Duct

On day 60, rats were euthanized using cardiac exsanguination under a surgical plane of ketamine and xylazine anesthesia. The liver and biliary duct specimens were excised for histopathologic assessment. Representative sections of all liver and biliary duct segments were embedded in paraffin after fixation of the tissues in 10% neutral buffered formalin. Five-micrometer sections were obtained from each paraffin block and stained with hematoxylin and eosin. Slides were examined in duplicate by a blinded pathologist under a light microscope (Nikon Eclipse E600, Nikon, Melville, New York). In hepatic tissue, hepatic parenchyma necrosis, hyperemia, portal inflammation, portal fibrosis, proliferation of biliary canaliculi, and cholangitis/pericholangitis, and in biliary duct tissues hyperemia and fibrosis were assessed under the light microscope. In each group, histopathologic alterations were scored as absent (0), slight (1), mild (2), and severe (3).[16]

Statistical Analysis

The numeric results were expressed as mean (SD). Severity scores were expressed as median (range) due to non-normal distribution. Normality of distribution of the variables was tested using the 1-sample Kolmogorov-Smirnov test. The Kruskal-Wallis test was used to compare differences between groups due to abnormal distribution, and the Mann-Whitney *U* test with Bonferroni correction was used if a significance result was obtained. A P value <0.05 was considered statistically significant. Statistica 7.0 (StatSoft Inc., Tulsa, Oklahoma) was used for statistical analyses.

RESULTS

In the 40 rats studied, no deaths occurred in the melatonin or control groups. Two rats died within 2 hours in the formalin group, and 1 rat died in the formalin + melatonin group after the drug administration. On necropsy, the main causes of death were considered iatrogenic portal vein catheterization and formalin injection through the catheter.

HISTOPATHOLOGIC EXAMINATION

Statistical values from the histopathologic examinations of the groups are shown in **Table I.** No significant histopathologic alterations were found in the melatonin or control group, with the exception of slight hyperemia in the control group (1 rat). On examination of the livers and biliary ducts in the formalin group, severe damage was found in 5 rats. Parenchymal necrosis in the liver, portal inflammation, portal fibrosis, biliary duct proliferation, and mild hyperemia were found in the formalin + melatonin group. Mild and slight hyperemia, cholangitis/pericholangitis, and fibrosis were noted in biliary duct sections in the formalin + melatonin group (**Figure** 1). Mean severity scores of parenchymal necrosis in the liver ($P < 0.01$), portal fibrosis ($P < 0.01$), biliary duct proliferation ($P < 0.001$), cholangitis/pericholangitis ($P < 0.01$), hyperemia in the biliary ducts ($P < 0.01$), and fibrosis ($P < 0.01$) were significantly lower in the formalin + melatonin group compared with the formalin group.

Table I. Histopathologic scores in this study of the effects of melatonin on caustic sclerosing cholangitis with scolicidal solution (formalin) in a rat model. Values are median (range).

Specimen	Formalin (n = 10)	Melatonin (n = 10)	Formalin + Melatonin (n = 10)	Control (n = 10)	P[a]
Hepatic parenchyma necrosis	1 (0–2)[b,c]	0 (0–0)	0 (0–0)[d]	0 (0–0)	<0.001
Hyperemia in liver	2 (1–3)[c,e]	1 (0–1)	1 (1–3)[f,g]	1 (1–1)	<0.001
Portal inflammation	1 (0–3)[b,c]	0 (0–0)	1 (0–1)[b,h]	0 (0–0)	<0.001
Portal fibrosis	1 (0–2)[b,c]	0 (0–0)	0 (0–1)[d]	0 (0–0)	<0.001
Proliferation of bile canaliculi	2 (1–3)[c,e]	0 (0–1)	0.5 (0–1)[f,i]	0 (0–0)	<0.001
Cholangitis/pericholangitis	2 (1–2)[c,e]	0 (0–1)	1 (0–1)[b,d,h]	0 (0–1)	<0.001
Hyperemia in biliary duct	2 (1–2)[c,e]	0 (0–1)	1 (1–1)[c–e]	0 (0–1)	<0.001
Fibrosis in biliary duct	2 (1–3)[c,e]	0 (0–1)	1 (0–2)[d,f,h]	0 (0–1)	<0.001

[a] Kruskal-Wallis test.
[b] $P < 0.01$ versus control.
[c] $P < 0.001$ versus melatonin.
[d] $P < 0.01$ versus formalin.
[e] $P < 0.001$ versus control.
[f] $P < 0.05$ versus control.
[g] $P < 0.05$ versus melatonin.
[h] $P < 0.01$ versus melatonin.
[i] $P < 0.001$ versus formalin.

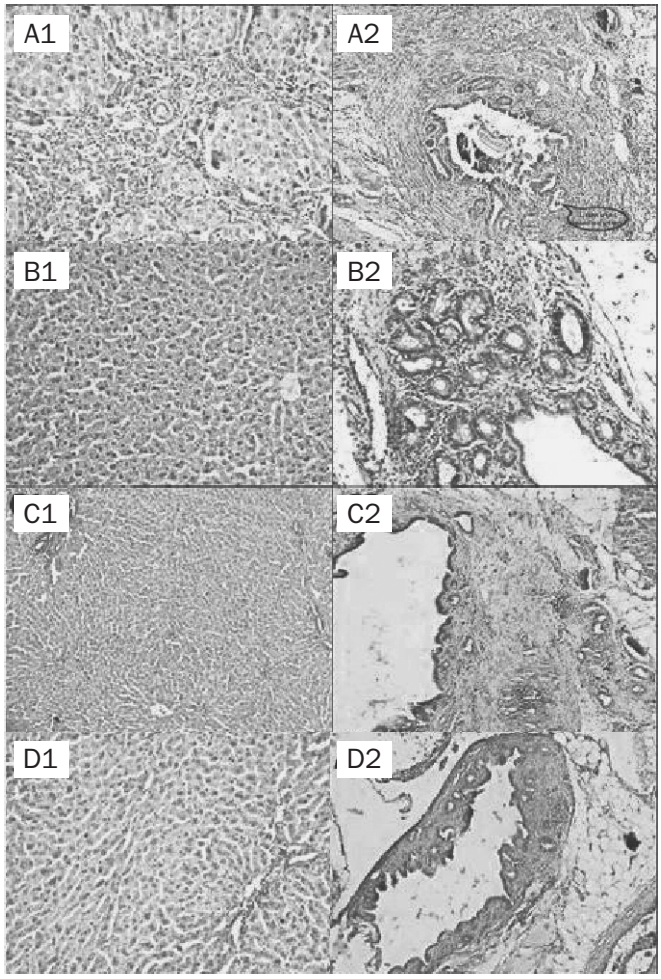


Figure 1. Histologic effects of melatonin on caustic sclerosing cholangitis with scolicidal solution (formalin) in a rat model. (A) Formalin group: (1) liver showing prominent ductular reaction and prominent reactive nuclear atypia in the hepatic cells (magnification, ×100); (2) biliary duct showing prominent inflammatory infiltration in and around biliary duct mucosa, and intraluminal leucocyte infiltration (×50). (B) Melatonin group: (1) liver showing minimal histology (×50); (2) biliary duct showing minimal chronic inflammation (×100). (C) Formalin + melatonin group: (1) liver showing minimal sinusoidal dilatation and minimal congestion of the portal vein (×50); (2) biliary duct showing minimal chronic inflammation (×50). (D) Control group: (1) liver showing normal histology (× 50); (2) biliary duct with normal histology (×50). (All staining, hematoxylin and eosin.)

HEPATOBILIARY SCINTIGRAPHIC EXAMINATION

Mean TPU and $t_{1/2}$ values in all 4 groups and the statistical analyses of the groups are shown in **Table II** and **Figure 2.** The TPUs in the control group did not differ significantly between the melatonin, formalin + melatonin, and control groups. A significant delay in TPU was found in the formalin group compared with controls ($P = 0.001$). No significant differences were observed between the formalin, melatonin, and formalin + melatonin groups with respect to $t_{1/2}$ values. The $t_{1/2}$ values were significantly longer in those groups than in the control group ($P < 0.001$).

Table II. Scintigraphic parameters in this study of the effects of melatonin on caustic sclerosing cholangitis with scolicidal solution (formalin) in a rat model. Values are mean (SE).

Parameter	Formalin (n = 10)	Melatonin (n = 10)	Formalin + Melatonin (n = 10)	Control (n = 10)	*P**
TPU, min (extraction)	17.2 (6.1)†	9.1 (2.7)	5.6 (0.5)‡	3.2 (0.1)	0.001
$t_{1/2}$, min (secretion)	48.3 (6.1)†	13.4 (7.4)†	44.1 (7.8)†	6.9 (0.2)	0.001

TPU = time of peak uptake.
*Kruskal-Wallis test.
† $P < 0.001$ versus control.
‡ $P < 0.001$ versus formalin.

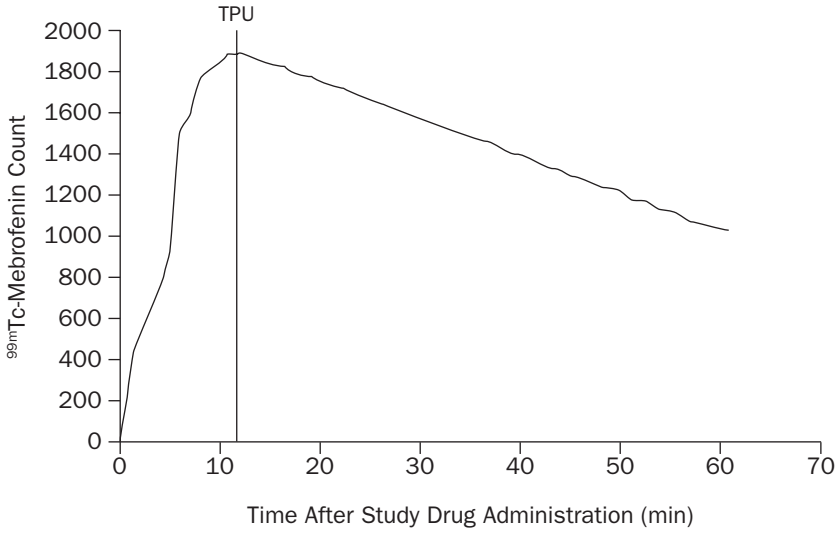


Figure 2. The time–activity curve of technetium-99m (^{99m}Tc)–mebrofenin in this study of the effects of melatonin on caustic sclerosing cholangitis with scolicidal solution (formalin) in a rat model. TPU = time of peak uptake (extraction).

DISCUSSION

This study used formalin, a scolicidal agent associated with caustic sclerosing cholangitis in a number of clinical and experimental studies.[8,10,12] When the data from the formalin group were compared to those from the controls, hyperemia in hepatic tissue, portal inflammation, portal fibrosis, proliferation in biliary ducts, and cholangitis, hyperemia, and fibrosis progression were detected at advanced stages in the biliary duct. In accordance with the literature, these data support the hypothesis that formalin might lead to caustic sclerosing cholangitis. The onset of scolicidal-induced sclerosing cholangitis was between 1 week and 1 year in humans.[12] Although different scolicidal agents are used in experimental models of caustic sclerosing cholangitis and histopathologic assessment is carried out at different time intervals, histopathology suggested caustic sclerosing cholangitis on study day 60. Critical histopathologic and scintigraphic results were also found, suggesting that contact between scolicidal agents (eg, formalin) and the intra- and extrabiliary ducts might lead to caustic sclerosing cholangitis.

The group administered formalin + melatonin was observed to have significantly less damage in terms of parenchymal necrosis in the liver ($P < 0.01$), portal fibrosis ($P < 0.01$), biliary duct proliferation ($P < 0.001$), cholangitis/pericholangitis ($P < 0.01$), hyperemia in the biliary ducts ($P < 0.01$), and fibrosis ($P < 0.01$) compared with the formalin group. Eşrefoğlu et al[16] reported a significantly protective effect of melatonin against fibrosis ($P < 0.05$), necrosis ($P < 0.05$), and portal inflammation ($P < 0.05$) of the liver in an experimental model of obstructive jaundice. In a study by Cruz et al,[20] the effects of melatonin were investigated in an experimental model of liver cirrhosis induced by thioacetamide. Liver injury was assessed using serologic analysis, as well as hematoxylin-eosin staining and the in situ apoptosis detection assay in liver sections. Oxidative stress was assessed using lipoperoxide and reduced glutathione levels, and by the measurement of catalase and superoxide dismutase activities in liver and serum. The study reported significantly less liver damage with thioacetamide with melatonin administration than without melatonin (lipoperoxide ($P < 0.01$), glutathione ($P < 0.01$), catalase ($P \leq 0.01$), and superoxide dismutase ($P \leq 0.01$). Padillo et al[19] reported that melatonin was associated with significantly less oxidative stress and hepatocyte destruction compared with controls in an experimental model of obstructive jaundice. In accordance with the literature,[16,17,19] data from the present study suggest that melatonin might attenuate the development of caustic sclerosing cholangitis resulting from scolicidal agent administration in the biliary ducts and liver.

The development of fibrosis is crucial in the progression of sclerosing cholangitis, and treatment protocols to impede this stage have been designed. Carossino et al[21] investigated the effects of melatonin in an experimental model of scleroderma. The authors assessed the growth rate based on growth curves and a 3H-thymidine incorporation assay. They reported that a dose of 200 µg/mL of melatonin attenuates (>80%) systemic sclerosis fibroblast activity. Those findings suggest that melatonin may attenuate the proliferation of fibroblasts derived from the skin of healthy subjects and patients with systemic sclerosis. A study of the attenuation of fibrosis formation

in rats was conducted by Hatipoğlu et al,[22] who reported favorable effects of melatonin on decreasing postoperative intra-abdominal adhesion formation. In an experimental design, 3 groups were studied—sham laparotomy, ischemia reperfusion, and ischemia reperfusion + melatonin. They assessed the effects of melatonin on intra-abdominal adhesion formation by blood glutathione peroxidase and microscopic and macroscopic adhesion scores. The authors reported that the glutathione peroxidase concentrations were significantly higher ($P = 0.026$) and fibroblast proliferation and macroscopic adhesion scores were significantly lower ($P = 0.001$), in the melatonin-treated group than in the melatonin-free group. They suggested that melatonin administration has beneficial effects on postoperative intra-abdominal adhesions. Consistent with the literature,[21,22] the present study found that the extent of fibrosis in the liver and biliary duct was significantly less in the formalin + melatonin group than in the formalin group ($P < 0.01$). Sahna et al[23] reported an elevation in congestive dilatation and mononuclear cell infiltration in the hepatic sinusoids of pinealectomized rats (ie, those with no endogenous melatonin). The authors investigated the effects of pinealectomy and administration of exogenous melatonin on liver tissue in rats. The structural changes and glutathione and malondialdehyde concentrations were assessed in the experimental design. Liver glutathione levels were significantly lower in pinealectomized rats than in the control group. Melatonin administration was associated with significantly increased glutathione concentrations ($P < 0.05$). Pinealectomy was associated with a significant increase in malondialdehyde concentrations compared with the control group ($P < 0.05$), and melatonin administration in pinealectomized rats was associated with significantly reduced malondialdehyde concentrations in the liver ($P < 0.05$). The authors suggested that a significant increase in oxidative and structural changes occur in rat livers after pinealectomy, and that these changes might be diminished by melatonin treatment. Messner et al[24] reported that the melatonin concentration in portal venous blood was higher compared with that in peripheral venous blood samples (by 20%–30%). A protective effect of melatonin, through the attenuation of oxidative stress and inflammation in thioacetamide-induced hepatic damage, was established in another experimental rat study.[25] The author reported that serum liver enzymes and blood ammonia were lower in rats treated with thioacetamide + melatonin compared with thioacetamide monotherapy ($P < 0.001$). Liver histology was significantly improved and the mortality in the melatonin-treated rats was significantly decreased ($P < 0.001$). Aust et al[26] assessed melatonin receptors located in human gallbladder epithelia and proposed that melatonin might have a protective effect on the biliary duct. On histopathology, formalin-induced biliary duct and hepatic damage might have been reduced with melatonin use. It appears that melatonin mediates this effect by eliminating oxidants released as a result of destruction developing with scolicidal agents in biliary duct epithelia by having an anti-inflammatory effect on released mediators or by attenuating fibrosis, which is crucial in the development of sclerosing cholangitis.[25] Further studies, supported by laboratory data, are necessary to elucidate this issue.

A review of the literature found in the MEDLINE, EMBASE, and Ovid databases, published from 1965 through 2009, found no studies on the topics including the

effect of melatonin on caustic sclerosing cholangitis due to scolicidal agents or assessments of the results on histopathology and scintigraphy. Because the literature search found no studies of the effects of scolicidal agents on the liver or biliary duct, or studies of prevention methods, no scintigraphic data were available. On scintigraphy in this study, no significant differences were observed between the melatonin, formalin + melatonin, and control groups with respect to TPU, whereas there were significant differences between the formalin group and the other groups. On scintigraphy, melatonin apparently provided protection for the extraction function of the hepatocytes in the liver against the damage caused by scolicidal agents. These findings were supported on histopathology. Although no significant differences were found between the formalin, melatonin, and formalin + melatonin groups on scintigraphy, there were significant differences between these groups and controls. This study did not find significant results that suggested that melatonin provides protection for the biliary secretory function against the damage caused by scolicidal agents. However, statistically significant differences versus controls suggested attenuating effects of melatonin on the extraction function of the hepatocytes.

Further experimental and clinical studies in humans, using scolicidal agents that do not cause as much tissue damage as does formalin, are needed to confirm the findings from this study of the effects of melatonin.

CONCLUSION

In this experimental study in a rat model of caustic sclerosing cholangitis, the histopathologic and scintigraphic findings suggested that melatonin is effective in attenuating the damage caused by scolicidal agents in the liver and biliary ducts.

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