INTRODUCTION

Melatonin, the chief secretory product of the pineal gland, is a free radical scavenger and antioxidant (Reiter et al., 1995). Tan et al. (1993a) initially reported that melatonin neutralized the highly toxic hydroxyl radical (·OH), an observation which has been confirmed by several authors (Matuszak et al., 1997; Susa et al., 1997; Stasica et al., 1998). Studies of Pieri et al. (1994, 1995) suggest that melatonin is also superior to vitamin E as a peroxyl radical (LOO·) scavenger although this observation has not been confirmed in vivo. Most recently, melatonin has been suggested to scavenge the peroxynitrite anion (ONOO−) (Cuzzocrea et al., 1998) as well as nitric oxide (NO·) (Noda et al., 1999). Furthermore, the indole has been shown to be highly effective in reducing lipid peroxidation (LPO) induced by a variety of toxicants (Reiter, 1995a,b, 1997). Indeed, melatonin has been extensively tested for its ability to resist oxidative processes both in vitro and in vivo. In this capacity, melatonin has been shown to combat oxidative damage not only to lipids (De La Lastra et al., 1997; Li et al., 1997; Melchiorri et al., 1997; Princ et al., 1997; Daniels et al., 1998) but also to DNA (Tan et al., 1993b, 1994; Susa et al., 1997).

Di Luzio (1963) was the first to observe that pretreatment with antioxidants alleviated hepatic fat accumulation in rats treated with ethanol. Di Luzio and Hartman (1967) also showed that ethanol addition in vitro to liver homogenates results in an increased LPO and that this is prevented by the simultaneous addition of antioxidants. Subsequently, other investigators found that both acute and chronic ethanol intoxication is associated
with an increase in LPO (Albano et al., 1991; Nordmann et al., 1992). It is known that alcohol abuse induces testicular damage leading to endocrine and reproductive dysfunction. Rosenblum et al. (1989) postulated that free radical generation and LPO might be an important mechanism in the toxicity of ethanol in the testes. In fact, these authors showed an increase in testicular levels of oxidatively damaged polyunsaturated fatty acids in rats fed alcohol chronically. Also a direct correlation was found between testicular atrophy and loss of an important antioxidant, reduced glutathione (GSH).

It has been postulated that free radicals play an important role in myocardial injury and this mechanism has been implicated in the pathogenesis of alcoholic cardiomyopathy (Edés et al., 1986). Chronic ethanol intake induces changes in components of the myocardial antioxidant defence system (Edés et al., 1986), such as GSH levels (Guerri and Grisolia, 1980), and in the cytosolic and membranous protein thiols (Ribiere et al., 1992), which could contribute to the increase in free radical formation in this tissue.

Yang and Carlson (1991) found significant decreases in GSH and glutathione-S-transferase (GST) activity in lungs of rats treated acutely with ethanol. Ethanol-induced fatty acid ethyl esters play a role in the development of alcohol-related injury to the lung (Manautou and Carlson, 1991).

The brain possesses certain characteristics which may make it especially prone to oxy-radical injury. Chronic ethanol administration is known to induce oxidative stress in the brain by increasing LPO in at least some brain regions (Nadiger et al., 1988) and enhancing the generation of oxygen radical species in synaptosomes of chronically alcohol-fed rats (Montoliu et al., 1994). In view of the findings summarized above and the newly reported role of melatonin as an antioxidant, the current study was designed to investigate the efficacy of melatonin in reducing the peroxidation of lipids in tissues known to be susceptible to alcohol toxicity, namely, the liver, brain, lung, heart and testes.

MATERIALS AND METHODS

Animals

Forty-one adult male Sprague–Dawley rats weighing ~150 g, purchased from Harlan (Houston, TX, USA), were used in this study. All rats were kept under the same laboratory conditions of temperature (22 ± 2°C), relative humidity (45 ± 5%) and light: dark cycle (14 light:10 h dark) and were allowed free access to tap water and standard food. The food provided was Teklad LM-485 Rat Sterilizable Diet which contains 103.80 IU/kg vitamin E and 34.36 IU/kg vitamin A.

Chemicals

Melatonin was purchased from Sigma (St Louis, MO, USA) and dissolved in ethanol before being diluted with saline. The final concentration of ethanol in the melatonin solution was <1%. The Bioxytech LPO-586 assay kit, purchased from Cayman Chemical (Ann Arbor, MI, USA), was used for measuring the products of LPO [malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA)]. All other chemicals were of the highest quality available.

Experimental design and procedures

The rats were divided into four groups. The first group (eight rats) served as controls and received a subcutaneous injection of saline (containing <1% ethanol). The second group (nine rats) was given melatonin only. The third group (12 rats) was given a daily subcutaneous injection (3 g/kg body weight) of 40% ethanol in saline solution only. The final group (12 rats) was given daily a similar injection of ethanol, which was preceded, 30 min earlier, by a subcutaneous melatonin injection; the daily dose of melatonin was 10 mg/kg body weight. All of these injections were repeated daily for 30 days; the melatonin or alcoholic saline injections were given at 18:00, 30 min before the administration of alcohol. At the end of the treatment period, the rats were killed by decapitation and the liver, heart, lung, brain and testes were removed, frozen on solid CO₂ and stored at −80°C.

MDA + 4-HDA concentrations are considered to be an index of the peroxidation of membrane lipids (Esterbauer and Cheeseman, 1990). The colorimetric kit mentioned above was used to determine the levels of oxidized lipid. At the time of assay, tissue from each organ was thawed, homogenized in ice-cold 50 mM Tris buffer (pH 7.4, 10% w/v) using an Euro Turrax T20b homogenizer and the supernatant prepared by centrifugation at 10 000 g for 10 min at 4°C; these were used to measure MDA and 4-HDA. Protein concentrations were
measured by the method of Bradford (1976) using bovine albumin as standard.

**Statistical analysis**

The data are presented as the arithmetic means ± SEM. Statistical analyses were performed using an ANOVA followed by the Student–Newman–Keuls t-test. $P < 0.05$ was considered to be significant. The percentage stimulation (S%) or inhibition (I%) in the mean values of LPO were calculated as follows.

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\text{Percentage stimulation (S%)} = \frac{(\text{mean ethanol value} - \text{mean control value})}{\text{mean control value}} \times 100
\]

\[
\text{Percentage inhibition (I%)} = \frac{(\text{mean ethanol + melatonin value} - \text{mean ethanol value})}{\text{mean ethanol value}} \times 100
\]

**RESULTS**

The mean final body weights (± SEM) of the four groups of rats (control, melatonin, ethanol, ethanol + melatonin) at the end of the experiment were 276 ± 6, 278 ± 4, 268 ± 7 and 272 ± 3 g, respectively. These values were not statistically significantly different.

The data are summarized in Figs 1–5. Chronic administration of ethanol increased LPO (as indicated by the increase in MDA + 4-HDA levels) in the testes (Fig. 2), heart (Fig. 3), lung (Fig. 4) and brain (Fig. 5) with no statistically significant change being observed in the liver (Fig. 1). The ethanol-induced increases in LPO were 21.8% in the brain and more than double that extent (45.3%) in the testes; both these increases were significant ($P < 0.05$). A significant stimulation of LPO was also found in the heart (28.8%) and the lungs (35.9%) (both with $P < 0.01$). When melatonin was administered to alcohol-treated rats, it reduced LPO levels to those measured in the control rats. Statistical analysis of these data indicate that melatonin significantly reduced ($P < 0.05$) LPO levels in the heart (19.7%) and testes (39.6%), respectively, while in the brain and lung the reductions were 31.3% and 28.1%, respectively (both $P < 0.01$) (Figs 2–5). Melatonin administration did not change the level of LPO products in the liver of rats treated with ethanol. Likewise, the daily injection of melatonin into non-ethanol-maintained rats did not change basal levels of MDA + 4-HDA in any of the organs studied.

![Fig. 1](image1.png)

Fig. 1. Effect of melatonin on malondialdehyde (MDA) + 4-hydroxyalkenal (4-HAD) levels (lipid peroxidation products) in the liver after chronic ethanol administration to rats.

Con, control rats; Mel, melatonin (10 g/kg daily); Eth, ethanol (3 mg/g daily).

![Fig. 2](image2.png)

Fig. 2. Inhibitory effect of melatonin on MDA + 4-HDA levels (lipid peroxidation products) in the testes after chronic ethanol administration to rats.

* $P < 0.05$ versus ethanol-treated rats. For abbreviations, see legend to Figure 1.
DISCUSSION

Ethanol is believed to generate oxygen radicals, inhibit GSH synthesis and deplete GSH levels in tissues, increase MDA levels and generally impair the antioxidative defence system in humans and experimental animals (Speisky et al., 1985; Gence et al., 1998). Marked decreases in the GSH pool occur in a variety of tissues after acute and chronic ethanol intoxication (Guerrì and Grisolia, 1980); this is partly a result of binding of cysteine and/or GSH by acetaldehyde (Lieber, 1994). Many workers have successfully reduced alcohol toxicity by antioxidant administration (Valenzuela et al., 1985; Rosenblum et al., 1987; Videla and Guerrì, 1990).

Ethanol-induced tissue damage occurs in a variety of organs, including the liver, where ethanol is actively oxidized; extrahepatic tissues in the rat also have been shown to exhibit oxidative damage following acute or chronic ethanol intoxication (Nordmann et al., 1990). Tissues such as the central nervous system, heart, lungs and testes are especially prone to ethanol-induced LPO, at least in vitro. These findings suggest that, in vivo, ethanol may likewise induce oxidative stress in a variety of organs.

Herein we observed a minor statistically insignificant rise (4%) in hepatic MDA + 4-HDA levels in rats treated with ethanol for 30 days (Fig. 1). Guerrì and Grisolia (1980) also did not observe changes in the hepatic oxidized glutathione levels after chronic ethanol administration. Likewise, conflicting results on the hepatic content of GSH have been reported after chronic ethanol administration to experimental animals. Some investigators found no change in GSH content, whereas others
observed either decreases or increases (Videla and Guerri, 1990).

Whereas the liver, in general, has a well-developed antioxidative defence system to protect against free radicals, more prolonged treatment with ethanol would probably have damaged the hepatocytes as well. Of interest is that we have recently found very high levels of melatonin in the bile of a variety of species (D. X. Tan, L. C. Manchester, R. J. Reiter, unpublished observations), even in animals not treated with the indole. The melatonin in the bile is likely to have been excreted by the liver, suggesting that the hepatocytes may normally contain higher amounts of this antioxidant than do most other organs. This may have helped to protect the liver from oxidative damage.

Ethanol is a known testicular toxin and its chronic use leads to both endocrine and reproductive failure (Rosenblum et al., 1989). Because testicular membranes are rich in polyenoic fatty acids that are prone to undergo peroxidative decomposition, it is likely that the resulting LPO contributes to the membrane injury and gonadal dysfunction that occurs as a result of alcohol abuse and/or chronic use. The present results (Fig. 2) show that testicular LPO is a metabolic consequence of chronic ethanol administration to animals. Herein we observed a significant stimulation (43.5%, \( P < 0.05 \)) of LPO levels in the testes of ethanol-treated rats compared with that in controls. This marked stimulatory effect of ethanol on LPO demonstrates that ethanol modifies the precarious antioxidant balance of testicular tissue such that enhanced peroxidation occurs. It is known that peroxidative injury in the testes can be attenuated by dietary vitamin A supplementation (Rosenblum et al., 1987). Melatonin, a molecule known to reduce the decomposition of membrane lipids due to a variety of toxicants (Reiter, 1995a,b, 1997), is also shown in the present study to inhibit ethanol-induced LPO.

The contribution of free radicals to heart disease is well documented (McCord, 1989) and oxidative stress has been suggested to represent a fundamental mechanism in the induction of myocardial injury (Thompson and Hess, 1986). The likelihood that free radical mechanisms leading to LPO are involved in cardiac damage during alcohol intoxication is supported by several reports (Redetzki et al., 1983; Edes et al., 1986; Panchenko et al., 1987). Moreover, many researchers (Preedy and Richardson, 1994; Ashakumary and Vijayammal, 1996; Figueredo, 1997) have found that excessive alcohol intake is a major risk factor for the development of cardiovascular disease. In the present study (Fig. 3), ethanol significantly stimulated (by 28.8%, \( P < 0.01 \)) LPO levels in the heart compared to those measured in controls; again, the rise was counteracted by melatonin given in advance of ethanol administration.

Ethanol-induced LPO in the heart may be mediated by an increase in the conversion of xanthine dehydrogenase to xanthine oxidase (Oei et al., 1982) and an enhancement of the activity of acyl CoA-oxidase (Panchenko et al., 1987), both of which generate free radicals. Changes in the levels of some antioxidant enzymes in cardiac tissue have also been observed after chronic alcohol treatment, suggesting an adaptive mechanism to the prooxidative challenge (Ribiere et al., 1992).

In the current study, ethanol significantly stimulated (21.8%, \( P < 0.05 \)) LPO levels in neural tissue (Fig. 5). Chronic ethanol intake decreases the levels of the GSH (Guerri and Grisolia, 1980; Montoliu et al., 1994) and \( \alpha \)-tocopherol content of the cerebellum (Rouach et al., 1991). In addition, ethanol intake is associated with adaptive changes in the antioxidant defence enzymes such as increased levels of neural superoxide dismutase and catalase (Montoliu et al., 1994).

An increase in the low molecular weight iron, which has been observed in the cerebellum of ethanol-treated rats, and its putative links to local ethanol metabolism may contribute to the enhanced generation of radicals (Rouach et al., 1991; Nordmann et al., 1992). Other studies have indicated that ethanol may be metabolized by catalase (Arago et al., 1992) and an ethanol-inducible form of cytochrome P-450 (CYP2E1) has been observed in various regions of the rat brain (Hansson et al., 1990). Moreover, Guerri et al. (1994) have observed that chronic ethanol intake induces a rise in the neural activity of total cytochrome P-450 as well as CYP2E1. The induction of CYP2E1 could increase the generation of pro-oxidant free radical species (e.g. OH), thereby decreasing GSH and \( \alpha \)-tocopherol levels leading to augmented oxidative stress in the brain of chronically alcohol-fed rats.

In this study, chronic ethanol administration caused a significant stimulation (35.9%, \( P < 0.01 \)) of lipid breakdown products in the lungs compared to those in controls (Fig. 4). Manautou and Carlson (1991) indicated that ethanol-induced fatty acid
ethyl esters (the end products of a non-oxidative pathway for ethanol metabolism) play a role in the development of alcohol-related injuries to the lung. A significant decrease in GSH and GST activity in the lung was found in rats treated acutely with ethanol (Yang and Carlson, 1991; Eke et al., 1996; Ashakumary and Vijayammal, 1996). Histological examination indicated cell damage, intravascular haemostasis and cell oedema in the lung after acute ethanol administration (Moriya et al., 1992). The results were taken to mean that cell damage and intravascular haemostasis were caused by a direct action of ethanol.

Reporting damaging effects of ethanol in the lung, Holguin et al. (1998) found that ethanol ingestion further decreased reduced GSH levels in lung tissue and lung lavage fluid, and increased oxidized glutathione levels in the lung lavage fluid. Furthermore, ethanol ingestion decreased type II cell GSH content, decreased type II cell surfactant synthesis and secretion and cell viability in vitro.

Melatonin reduced the products of LPO induced by chronic ethanol exposure with seemingly equal efficacy in all organs studied. This suggests that melatonin is taken up by each of these organs. Certainly, a well-known feature of melatonin is the ease with which it passes all morphophysiological barriers and enters cells. The few studies that have been performed have shown that exogenously administered melatonin quickly gets into cells throughout the organism, with only seemingly minor differences between organs in terms of the quantity of melatonin taken up (Menendez-Pelaez et al., 1993). Interestingly, however, the highest melatonin concentrations seem to be in the nuclei of cells, rather than in the lipid-rich membranes.

None of the treatments altered the growth rate of the rats as indicated by the fact that final mean body weights did not differ among the experimental groups. As a result, it seems unlikely that the level of LPO in the organs studied was a consequence of antioxidants taken in through the diet, e.g. vitamin E.

It is now well documented that melatonin, a secretory product of the pineal gland, is capable of preventing oxidative damage (Reiter et al., 1995). That antioxidants reduce oxidative damage in the lungs is established. Valenzuela et al. (1985) found that antioxidants, given prior to ethanol, abolished both hepatic oxidized glutathione accumulation and the increase in lipid breakdown products. In the current study, melatonin reduced LPO in the lungs of chronically ethanol-treated rats. Statistical analysis indicated that the melatonin inhibition of ethanol-induced LPO was significant in the brain and lung (P < 0.01) as well as in the heart and testes (P < 0.05), as shown in Figs 2–5.

Several groups have shown that melatonin efficiently scavenges ‘OH (Tan et al., 1993a; Matuszak et al., 1997; Stasica et al., 1998) and there is evidence that melatonin also scavenges ONOO− (Gilad et al., 1997; Cuzzocrea et al., 1997, 1998) and NO˙ (Noda et al., 1999). Both ‘OH and ONOO− can initiate LPO. Several studies have suggested that melatonin may also neutralize another damaging radical, the LOO˙, although the efficiency with which it does so is debated. The LOO˙ is formed during the process of LPO (Chance et al., 1979). Regardless of its questionable efficiency as a LOO˙ scavenger, melatonin has been shown to be very effective in reducing LPO induced by a variety of toxicants (Reiter, 1995a,b, 1997). This suggests that melatonin concentrations are sufficiently high in lipid-rich membranes to limit either directly or indirectly the peroxidation of lipids by free radicals. The reported subcellular distribution of melatonin is consistent with its high lipid solubility (Costa et al., 1995).

Besides functioning as free radical scavenger and antioxidant (Reiter, 1995a,c, 1997), melatonin also functions as a hormone (Reiter, 1995c) and immune system regulator (Maestroni, 1993). Whether these latter two functions of melatonin played a role in reducing oxidative destruction of lipid in the current study remains unknown. Even if these actions of melatonin were involved, however, it would seem likely that the ultimate mechanism still probably involved the detoxification of free radicals.

Many free radical-generating toxicants have been used to induce oxidative damage to lipids and in each case melatonin has proven effective in attenuating the molecular destruction. Some of the most toxic agents which have been utilized and shown to be counteracted by melatonin include the excitatory neurotransmitter analogue kainic acid (Melchiorri et al., 1995; Giusti et al., 1995, 1996), H2O2 (Chen et al., 1995; Sewerynek et al., 1995b), lipopolysaccharide (Sewerynek et al., 1995a,c,d), amyloid-β protein (Daniels et al., 1998), and the carcinogen safrrole (Tan et al., 1993b, 1994). In the present study, the quantity of melatonin administered caused blood levels of the indole (and therefore presumably tissue concentrations as well) to
exceed those obtained from endogenous sources, i.e. the doses were pharmacological. There is evidence, however, that even physiological levels of melatonin derived from the pineal gland are capable of protecting against oxidative stress (Tan et al., 1994; Manev et al., 1996). Taken together, the results of the present study along with the data showing that blood melatonin levels drop in advanced age, suggest that alcohol toxicity, at least in terms of LPO, may be greater in old rather than in young subjects.

REFERENCES


Sewerynek, E., Melchiorri, D., Chen, L. D. and Reiter, R. J. (1995a) Melatonin reduces both basal and bacterial


