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Suppressive effect of melatonin administration on ethanol-induced gastroduodenal injury in rats *in vivo*

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1 Melatonin protection against ethanol-induced gastroduodenal injury was investigated in duodenum-ligated rats.

2 Melatonin, injected i.p. 30 min before administration of 1 ml of absolute ethanol, given by gavage, significantly decreased ethanol-induced macroscopic, histological and biochemical changes in the gastroduodenal mucosa.

3 Ethanol-induced lesions were detectable as haemorrhagic streaks. Ethanol administration damaged 36% and 25% of the total gastric and duodenal surface, respectively. Melatonin treatment reduced ethanol-induced gastric and duodenal damage to 14% and 8%, respectively. When indomethacin was given together with ethanol, the gastric damaged area was 44% of the total surface, while the duodenal damaged area was 35%; melatonin administration reduced the damage to only 13% of the total gastric surface and to 12% of total duodenal surface.

4 Both stomach and duodenum of ethanol-treated animals showed polymorphonuclear leukocyte (PMN) infiltration. The number of PMN increased more than 600 and 200 times in stomach and duodenum, respectively, following ethanol administration. Melatonin treatment reduced ethanol-induced PMN infiltration by 38% in the stomach and 20% in the duodenum. In indomethacin-ethanol-treated rats, the number of PMN increased by 875% compared to control group in the stomach and by 264% in duodenum. Melatonin administration reduced the indomethacin-ethanol-induced PMN rise by 57% in the stomach and 40% in the duodenum.

5 Gastroduodenal total glutathione (tGSH) concentration and glutathione reductase (GSSG-Rd) activity were significantly reduced following ethanol and indomethacin-ethanol administration. Melatonin ameliorated both the decrease in tGSH concentration as well as the reduction of GSSG-Rd activity elicited by ethanol both in the stomach and duodenum; melatonin was effective against indomethacin-ethanol-induced damage only in the stomach.

6 Ethanol-induced gastroduodenal damage is believed to be mediated by the generation of free radicals. Recently, a number of *in vivo* and *in vitro* experiments have shown melatonin to be an effective antioxidant and free radical scavenger; thus, we conclude that the protection by melatonin against ethanol-induced gastroduodenal injury is due, at least in part, to its radical scavenging activity.

Keywords: Melatonin; ethanol; erosion; stomach; duodenum; free radicals; GSH; GSSG-Rd

Introduction

Ethanol consumption is one of the co-factors predisposing to acute gastroduodenal injury in man (Lulu & Dragstedt, 1970). Intragastric administration of ethanol to pylorus-ligated rats rapidly induces gastric mucosal lesions which are commonly used to study both the pathogenesis and therapy of human ulcerative disease. Ethanol rapidly penetrates the gastroduodenal mucosa causing membrane damage, exfoliation of cells and erosion. The subsequent increase in mucosal permeability together with the release of vasoactive products from mast cells, macrophages and other blood cells may lead to vascular injury, necrosis and ulcer formation (Szabo, 1987).

It has been suggested that one of the mechanisms responsible for ethanol-induced gastroduodenal damage is the generation of free radicals (Pihan *et al*., 1987; Szelenyi & Brune, 1988). Superoxide dismutase and catalase, two endogenous antioxidants which protect cells against superoxide anion and hydroperoxide, respectively, decrease mucosal damage caused by ethanol administration (Pihan *et al*., 1987). Compounds with free radical scavening properties such as thiourea, dimethylsulphoxide and sulphydryl-containing substances significantly reduce ethanol toxicity to gastric mucosa, whereas naturally occurring antioxidants, including α-tocopherol and β-carotene are ineffective (Szelenyi & Brune, 1988). Also, oxygen-derived free radicals have been shown to participate in reperfusion damage both in the intestine and stomach leading to lesions which morphologically resemble those induced by ethanol (Parks *et al*., 1982; 1983; Itoh & Guth, 1985).

Recently, the pineal hormone melatonin was shown to scavenge both the hydroxyl and peroxyl radical (Tan *et al*., 1993; Pieri *et al*., 1994; Poeggeler *et al*., 1995). Both *in vitro* and *in vivo* studies have demonstrated the antioxidant properties of melatonin (Hardeland *et al*., 1995; Reiter *et al*., 1995; Reiter, 1995). The indolamine confers protection against oxidative stress caused by different chemicals in several tissues and biological systems (Abe *et al*., 1994; Tan *et al*., 1994; Melchiorri *et al*., 1995a,b; Sewerynek *et al*., 1995a,b,c).

In the present study, we investigated the potential protective effect of melatonin against ethanol-induced gastroduodenal injury in duodenum-ligated rats. Ethanol-induced mucosal damage was evaluated by three different approaches: macroscopic investigation, histological analysis and measurement of biochemical parameters.

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Methods

Animals

Eighty four Sprague Dawley male rats (225–250 g) were obtained from Harlan (Houston, TX) and housed in Plexiglas cages with 3 animals per cage. The animal rooms were windowless with automatic temperature ($22 \pm 1^{\circ}C$) and lighting controls (light on at 07h 00min and off at 21h 00 min; 14 h light/10 h dark). The rats received standard laboratory chow and water *ad libitum*.

Animal treatment

Forty eight hours before the experiments, the rats were fasted and allowed access to water *ad libitum*. They were kept in cages with wide mesh wire bottoms to prevent coprophagia. On the day of the experiment, animals were anaesthetized with 'rodent cocktail' (ketamine 60 mg ml^{-1} + xylazine 8 mg ml^{-1}) at a dose of 0.2 ml/250 g^{-1} body weight and the duodenum was ligated at a site 2 cm distal to the pylorus. After ligation, rats were divided in 5 groups of 12 animals each and 2 groups (control and melatonin groups) of 10 animals each. In previous experiments undertaken in our laboratory, ethanol injection at a dose of 2.3% i.p. was not protective against gastroduodenal injury induced by the intragastric administration of 1 ml of absolute ethanol. This observation is consistent with the knowledge that low doses of ethanol injected i.p. stimulate gastric acid secretion, thus inducing gastric damage (Glass *et al*., 1979). On the basis of these data, in the present study the control group was not injected with 2.3% ethanol and received only 1 ml of distilled water by gavage with a metal orogastric tube. Group 2 received 1 ml of absolute ethanol by gavage. Group 3 was treated with melatonin (10 mg kg^{-1}, i.p.) followed, 30 min later, by ethanol. Group 4 was injected with melatonin and received 1 ml of distilled water intragastrically. Group 5 was injected with indomethacin s.c. and, after 30 min, received ethanol by gavage. Group 6 was treated with melatonin and indomethacin and, after 30 min, the animals received ethanol intragastrically. Group 7 received indomethacin s.c. and distilled water by gavage. One hour after the administration of ethanol, animals were killed by decapitation. The stomach and duodenum were removed. The stomach was opened along the greater curvature and washed, together with duodenum, in physiological saline.

Macroscopic and histological analysis

For the measurement of gross gastric lesions, the freshly excised stomach was laid flat and the glandular and non-glandular portions of the stomach were outlined and the mucosal lesions were traced on clear acetate paper. Gross mucosal lesions were recognized as haemorrhage or linear breaks (erosions) with damage to the mucosal surface; the induced haemorrhage caused blood to coagulate on the mucosal surface. After photographic enlargement, the area of gross lesions was calculated in a blind manner by planimetry. The glandular stomach was separated from the non-glandular portion and the damaged part was expressed as the percentage of either the total gastric area or of the non-glandular area. The same procedure was followed for the measurement of gross duodenal lesions. For histological examinations, stomach and duodenum were washed in phosphate buffer and dehydrated in graded concentrations of ethanol (70, 80, 90 and 100%); the tissues were embedded in paraffin. In the case of the stomach, histological sections were cut along the longitudinal axis so they contained portions of both the glandular and non-glandular stomach. The duodenum was cut to obtain cross-sections. From each sample, 4 μm thick sections were obtained and stained with haematoxylin-eosin to evaluate morphological damage. Gastroduodenal damage was scored for each histological section as follows: score 0, no lesions; 1, diffuse hyperaemia; 2, one or two haemorrhagic lesions or erosions; 3, three to five haemorrhage lesions or erosions; 4, more than 5 haemorrhagic lesions or erosions; 5, 20 to 40% of total gastroduodenal surface with haemorrhagic lesions or multiple erosions; 6, more than 40% of total gastroduodenal surface with haemorrhagic lesions or multiple erosions. Polymorphonuclear leukocytes (PMNs) were counted in 20 microscopic fields ($\times 250$).

Measurement of biochemical parameters

For biochemical examinations, both stomach and duodenum were frozen on solid CO_2 and stored at $-80^{\circ}C$ until the day of the assay (no more than 3 days after decapitation). All samples were obtained at approximately the same time of the day (between 09h 00min and 12h 00min) because of the diurnal variation in the concentration of sulphydryl compounds in tissues (Beck *et al*., 1958). Total GSH (tGSH) and oxidized glutathione (GSSG) were measured by the method of Griffith (1985). Glutathione peroxidase (GSH-Px) and GSSG-Rd were measured according to Jaskot *et al*. (1983) and Goldberg and Spooner (1985), respectively.

Chemicals

All chemicals were of the highest purity available. Absolute ethanol, melatonin, indomethacin hydrochloride, saturated picric acid, NADPH tetrasodium salt, 5-5′-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH) and glutathione reductase (GSSG-Rd) were obtained from Sigma (St. Louis, MO). 2-Vinyl-pyridine monomer was purchased from Fluka (Ronkonkoma, N.Y.). Melatonin was dissolved in absolute ethanol (the alcohol concentration in the final solution was 2.3%) and administered i.p. as a single dose of 10 mg kg^{-1}. Indomethacin hydrochloride was dissolved in saline and administered subcutaneously as a single dose of 5 mg kg^{-1}.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA). If the F values were significant, Student-Newman-Keul's test and Mann Whitney test were used to compare the melatonin-ethanol group vs ethanol group, the melatonin-indomethacin-ethanol group vs indomethacin-ethanol group and each group vs control and melatonin group. Linear regression was calculated according to Winer (1971). The level of significance was accepted at $P < 0.05$.

Results

Macroscopic and histological results

In the stomach, macroscopically, the gastric damaged areas were oedematous with lesions detectable as haemorrhagic streaks. Ethanol administration damaged 36% of total gastric surface, with the damaged area in the non-glandular stomach being only 5% (Table 1). Melatonin pretreatment reduced the damaged area to 14% and 2% of total gastric surface and non-glandular surface, respectively. Indomethacin-ethanol treatment increased the damage both in the total gastric surface as well as in the non-glandular surface (Table 1). Melatonin administration significantly protected the glandular as well as the non-glandular stomach (Table 1). Histologically, the damaged areas included exfoliation and necrosis of superficial cells, an oedematous submucosa, PMN infiltration and erosions. One hour after ethanol and indomethacin-ethanol administration, there was a significant improvement of the lesion scores because the damage was partially counteracted by melatonin treatment (Table 2). Melatonin administration greatly decreased the area of damage both in ethanol (Table 2) and in indomethacin-ethanol-treated rats (Table 2). Also, melatonin reduced the number of PMNs in ethanol and indomethacin-ethanol groups (Table 3).

Table 1 Melatonin protection against ethanol and ethanol plus indomethacin-induced macroscopic gastroduodenal damage

	Control	*ETOH*	*ETOH + MLT*	*ETOH + Indo*	*ETOH + Indo + MLT*	*MLT*
% total gastric surface	1.2±0.02	36±0.03$^{\circ\circ\circ}$	14.3±0.02***$^{\circ\circ\circ}$	44.5±0.04$^{\circ\circ\circ}$	13.5±0.04$^{+++\circ\circ\circ}$	1.2±0.02
% non-glandular gastric surface	1.1±0.02	5±0.03$^{\circ\circ}$	2.1±0.02**$^{\circ\circ}$	9.1±0.03$^{\circ\circ\circ}$	2.2±0.03$^{++\circ\circ}$	1.3±0.01
% total duodenal surface	0.5±0.02	25±0.5$^{\circ\circ\circ}$	8.0±0.05**$^{\circ\circ\circ}$	35±0.5$^{\circ\circ\circ}$	12±0.4$^{++\circ\circ}$	0.6±0.02

The damaged area is expressed as percentage of total surface. Data were analysed by one way analysis of variance followed by Student-Newman-Keul's test. Data are the means±s.e.mean. $^{\circ\circ\circ}P<0.001$, $^{\circ\circ}P<0.01$ vs control and melatonin groups; ***$P<0.001$, **$P<0.01$ vs ethanol group; $^{+++}P<0.001$, $^{++}P<0.01$ vs ethanol-indomethacin-group. ETOH, ethanol; MLT, melatonin; Indo, indomethacin.

Table 2 Histological evaluation of melatonin protection against ethanol and ethanol plus indomethacin-induced gastroduodenal damage

	Control	*ETOH*	*ETOH + MLT*	*ETOH + Indo*	*ETOH + Indo + MLT*	*MLT*
Total stomach	0	4.6±0.24	2.8±0.2**	6.0±0.2	3.0±0.3^{++}	0
Non-glandular stomach	0	1.6±0.24	0.6±0.2*	2.6±0.2	0.8±0.3^{++}	0
Duodenum	0	4.7±0.3	1.4±0.24**	5.6±0.2	3.0±0.3^{++}	0

For each stomach and duodenum, 10 histological sections were examined. Data were analysed by Mann Whitney test. Data are the means±s.e.mean. *$P<0.05$, **$P<0.01$ vs ethanol group; $^{++}P<0.01$ vs ethanol plus indomethacin-group. ETOH, ethanol; MLT, melatonin; Indo, indomethacin.

Table 3 Melatonin protection against ethanol and ethanol plus indomethacin-induced polymorphonuclear leukocyte (PMN) infiltration

	Control	*ETOH*	*ETOH + MLT*	*ETOH + Indo*	*ETOH + Indo + MLT*	*MLT*
Stomach	115±7	856±22$^{\circ\circ\circ}$	529±17***$^{\circ\circ\circ}$	1,122±26$^{\circ\circ\circ}$	480±10$^{+++\circ\circ\circ}$	126±8
Duodenum	132±4	425±12$^{\circ\circ\circ}$	340±10***$^{\circ\circ\circ}$	481±11$^{\circ\circ\circ}$	287±6$^{+++\circ\circ}$	117±9

PMNs were counted in 20 microscopic fields. Data were analysed by one way analysis of variance followed by Student-Newman-Keul's test. Data are means±s.e.mean. $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ vs control and melatonin groups; ***$P<0.001$ vs ethanol group; $^{+++}P<0.001$ vs ethanol-indomethacin-group. ETOH, ethanol; MLT, melatonin; Indo, indomethacin.

In the duodenum, ethanol and indomethacin-ethanol treatment damaged 25% and 35% of total duodenal surface, respectively (Table 1). Melatonin treatment reduced the damaged area to 8% and 12%, respectively (Table 1). Histologically, the duodenum of ethanol and indomethacin-ethanol-treated rats showed visible hyperaemia, exfoliation of superficial cells, denudation of villus epithelium, reduction of villus height, petechiae and epithelial necrosis with generalized blood cell infiltration. Melatonin administration decreased the area of the damaged surface. Ethanol and indomethacin-ethanol administration increased the duodenal lesion scores with melatonin being significantly protective (Table 2). Moreover, melatonin decreased PMN infiltration both in ethanol and indomethacin-ethanol groups (Table 3).

Biochemical parameters

In glandular stomach, ethanol administration reduced tGSH concentrations by 62% compared to control animals (Table 4). When rats were pretreated with melatonin, the decrease in tGSH was only 35% (Table 4). Indomethacin did not increase the loss in tGSH elicited by ethanol alone (Table 4); the protection conferred by melatonin against indomethacin-ethanol-induced biochemical changes was less effective compared to its action against ethanol alone (Table 4). Neither ethanol nor indomethacin-ethanol treatments altered the concentration of GSSG (Table 4). Also, melatonin alone did not alter the tGSH status (Table 4). Ethanol by itself decreased tGSH content of the non-glandular stomach by 42% compared to that in control animals (Table 4). The decrease was partially reversed by melatonin treatment (Table 4). Pretreatment with indomethacin reduced the non-glandular stomach tGSH by 60% (Table 4) with melatonin partially preventing the loss in tGSH (Table 4). Neither ethanol nor indomethacin + ethanol changed the concentrations of GSSG (Table 4). Ethanol, when administered alone, reduced duodenal tGSH by 37% compared to control animals with melatonin partially reversing this effect (Table 4). Indomethacin injection did not exaggerate the reduction in tGSH concentration (Table 4). No protection was afforded by melatonin when rats were pretreated with indomethacin (Table 4). Neither ethanol nor indomethacin-ethanol changed the duodenal concentration of GSSG (Table 4).

Gastroduodenal GSH-Px activity remained unchanged in ethanol as well as in indomethacin-ethanol-treated rats (Table 5). Melatonin, in combination with ethanol, increased GSH-Px activity in the non-glandular stomach and duodenum, but not in the glandular region of the stomach (Table 5).

GSSG-Rd activity was significantly decreased by ethanol administration both in the stomach and duodenum (Table 5) with melatonin completely reversing this effect. Indomethacin administration did not exaggerate the loss in enzyme activity induced by ethanol (Table 5) but it decreased the protection afforded by melatonin in the glandular stomach (Table 5) and completely prevented it in the duodenum (Table 5). Melatonin completely protected the non-glandular stomach against the loss in the enzyme activity (Table 5).

When macroscopic and histological data were fitted together with biological data by linear regression analysis, a direct

Table 4 Melatonin protection against ethanol and ethanol plus indomethacin-induced changes in (A) total reduced glutathione (tGSH) and (B) oxidized glutathione (GSSG) concentrations

	Glandular stomach	*Non-glandular stomach*	*Duodenum*
A		*tGSH* (μmol g^{-1} tissue)	
Control	1.41 ± 0.01	0.80 ± 0.08	0.87 ± 0.03
ETOH	$0.53 \pm 0.07^{\circ\circ\circ}$	$0.46 \pm 0.02^{\circ\circ\circ}$	$0.55 \pm 0.05^{\circ\circ\circ}$
ETOH-MLT	$0.93 \pm 0.15^{**\circ\circ}$	$0.66 \pm 0.07^{*\circ\circ}$	$0.78 \pm 0.02^{**}$
ETOH-Indo	$0.53 \pm 0.06^{\circ\circ\circ}$	$0.30 \pm 0.03^{\circ\circ\circ}$	$0.51 \pm 0.06^{\circ\circ\circ}$
ETOH-Indo-MLT	$0.78 \pm 0.03^{+\circ\circ}$	$0.48 \pm 0.03^{+++\circ\circ}$	$0.61 \pm 0.05^{\circ\circ}$
MLT	1.43 ± 0.05	0.90 ± 0.10	0.90 ± 0.04
B		*GSSG* (nmol g^{-1} tissue)	
Control	31.6 ± 1.3	18.0 ± 0.6	20.8 ± 1.4
ETOH	29.8 ± 0.6	20.9 ± 1.1	20.3 ± 1.2
ETOH-MLT	33.8 ± 1.4	17.2 ± 1.1	21.1 ± 1.6
ETOH-Indo	29.8 ± 0.6	20.8 ± 0.7	20.8 ± 0.5
ETOH-Indo-MLT	28.0 ± 0.7	19.9 ± 0.8	18.3 ± 0.5
MLT	31.0 ± 0.9	19.0 ± 0.4	19.0 ± 0.8

Data were analysed by one way analysis of variance followed by Student-Newman-Keul's test. Data are the means $\pm$ s.e.mean. $^{**}P<0.01$, $^{*}P<0.05$ vs ethanol group; $^{+++}P<0.001$, $^{+}P<0.05$ vs ethanol-indomethacin-group; $^{\circ\circ\circ}P<0.001$, $^{\circ\circ}P<0.01$ vs control and melatonin groups. ETOH, ethanol; MLT, melatonin; Indo, indomethacin.

Table 5 Melatonin protection against ethanol and ethanol plus indomethacin-induced changes in (A) glutathione peroxidase (GSH-Px) and (B) glutathione reductase (GSSG-Rd) activity

	Glandular stomach	*Non-glandular stomach*	*Duodenum*
A		*GSH-Px* (μM NADPH oxidized min^{-1} mg^{-1} protein)	
Control	0.25 ± 0.04	0.13 ± 0.01	0.14 ± 0.01
ETOH	0.24 ± 0.03	0.12 ± 0.01	0.13 ± 0.01
ETOH-MLT	0.30 ± 0.02	$0.21 \pm 0.03^{*}$	$0.17 \pm 0.01^{*}$
ETOH-Indo	0.29 ± 0.02	0.10 ± 0.01	0.12 ± 0.02
ETOH-Indo-MLT	0.28 ± 0.02	0.17 ± 0.04	0.15 ± 0.02
MLT	0.27 ± 0.05	0.14 ± 0.10	0.16 ± 0.03
B		*GSSG-Rd* (u mg^{-1} protein)	
Control	9.7 ± 0.1	6.6 ± 0.9	21.8 ± 1.8
ETOH	$6.6 \pm 0.6^{\circ\circ}$	$2.5 \pm 0.2^{\circ\circ\circ}$	$16.8 \pm 0.7^{\circ}$
ETOH-MLT	$9.5 \pm 0.8^{*}$	$4.8 \pm 0.2^{***}$	$23.5 \pm 0.9^{**}$
ETOH-Indo	$5.8 \pm 0.2^{\circ\circ\circ}$	$3.3 \pm 0.6^{\circ\circ\circ}$	$16.4 \pm 1.0^{\circ\circ}$
ETOH-Indo-MLT	$7.6 \pm 0.6^{+\circ}$	$6.3 \pm 0.9^{+}$	$16.3 \pm 2.4^{\circ}$
MLT	10.0 ± 0.9	7.0 ± 0.4	20.0 ± 0.8

Data were analysed by one way analysis of variance followed by Student-Newman-Keul's test. Data are the means $\pm$ s.e.mean. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ vs ethanol group; $^{+}P<0.05$ vs ethanol-indomethacin-group; $^{\circ\circ\circ}P<0.001$, $^{\circ\circ}P<0.01$, $^{\circ}P<0.05$ vs control and melatonin group. ETOH, ethanol; MLT, melatonin; Indo, indomethacin.

correlation between ethanol-induced gastroduodenal mucosal damage and alteration in biochemical parameters was evident (Figures 1 and 2). Also, melatonin protection against ethanol-induced alteration of macroscopic parameters appeared to be linearly correlated with the protection afforded by the pineal hormone at the biochemical level both in the stomach and duodenum (Figure 1A and Figure 2A). Moreover, in the duodenum, melatonin-ethanol administration increased GSSG-Rd activity at a level significantly higher than that detected in the control group (Figure 2A). When indomethacin was administered together with ethanol, a direct correlation between indomethacin-ethanol-induced mucosal gross damage and alteration in tGSH concentration as well as in GSSG-Rd activity was evident only in the stomach (Figures 1B and 2B).

Discussion

Results from these experiments clearly show a significant protection by melatonin against ethanol-induced gastroduodenal injury. Ethanol damage to the gastroduodenal mucosa has been described by several investigators (Katon & Smith, 1973; Eastwood & Kirchner, 1974; Szabo, 1987). Both changes in the structure of the gastroduodenal mucosa as well as gastric loss of tGSH have been demonstrated (Tarnawski *et al*., 1981; Szabo, 1981; Lacy & Ito, 1982). Recently, indirect evidence has indicated that generation of oxygen free radicals following ethanol administration may be causative in these lesions (Del Maestro *et al*., 1981; Pihan *et al*., 1987; Shaw *et al*., 1990).

In the present study, melatonin administration before ethanol treatment greatly reduced the macroscopic and histological gastroduodenal damage. Moreover, melatonin significantly reduced PMN infiltration both in the stomach and duodenum. PMN infiltration is a constant feature in inflammation. The respiratory burst of activated PMNs causes the univalent enzymatic reduction of molecular oxygen to the superoxide anion radical (Baboir *et al*., 1973) which, in turn, can give rise to further generation of more reactive oxygen species (Haber & Weiss, 1934), thus increasing and propagating the damage. The reduction of PMN infiltration

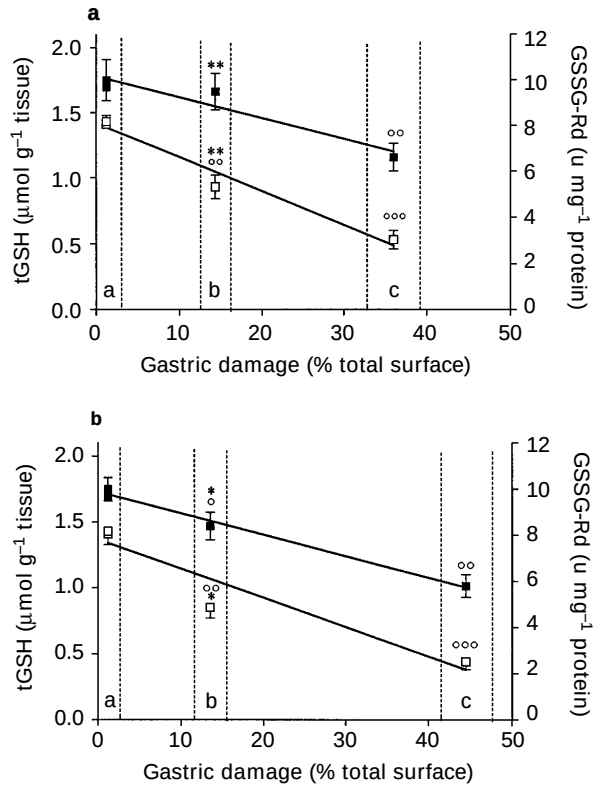

Figure 1 Correlation between gastric gross damage and gastric biochemical parameters following ethanol (A) and indomethacin plus ethanol (B) administration. Data were fitted by linear regression. (□) gastroduodenal total glutathione (tGSH); (■) glutathione reductase (GSSG-Rd) (A). Ethanol treatment: upper line = correlation between gastric damage and (■) GSSG-Rd activity ($r^2 = 0.96$; $F(1,2) = 47.66$; $P < 0.05$). Lower line = correlation between gastric damage and (□) tGSH concentration ($r^2 = 0.9673$; $F(1,2) = 59.22$; $P < 0.05$). Dotted lines delimit confidence interval of gastric damage: a = control and melatonin groups; b = ethanol-melatonin group; c = ethanol group. (B) Indomethacin plus ethanol treatment: upper line = correlation between gastric damage and (■) GSSG-Rd activity ($r^2 = 0.99$; $F(1,2) = 194$; $P < 0.01$). Lower line = correlation between gastric damage and (□) tGSH concentration ($r^2 = 0.91$; $F(1,2) = 19.47$; $P < 0.05$). Dotted lines delimit confidence interval of gastric damage: a = control and melatonin groups; b = indomethacin-ethanol-melatonin group; c = indomethacin-ethanol group. Biochemical data were analysed by one way ANOVA followed by Student-Newman-Keul's test. Each symbol represents the mean and vertical lines show s.e.mean. $^{*}P < 0.05$; $^{**}P < 0.01$ vs ethanol group; $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$ vs control and melatonin groups.

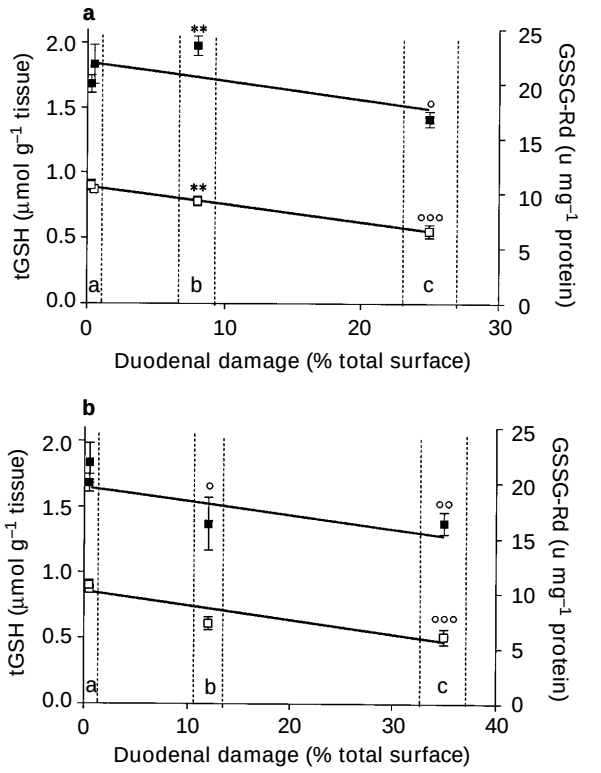

Figure 2 Correlation between duodenal gross damage and duodenal biochemical parameters following ethanol (A) and indomethacin plus ethanol (B) administration. Data were fitted by linear regression. (□) gastroduodenal total glutathione (tGSH); (■) glutathione reductase (GSSG-Rd). (A) Ethanol treatment: upper line = correlation between duodenal damage and (■) GSSG-Rd activity ($r^2 = 0.48$; $F(1,2) = 1.822$; $P > 0.05$). Lower line = correlation between duodenal damage and (□) tGSH concentration ($r^2 = 0.99$; $F(1,2) = 401.0$; $P < 0.01$). Dotted lines delimit confidence interval of duodenal damage: a = control and melatonin groups; b = ethanol-melatonin group; c = ethanol group. (B) Indomethacin plus ethanol treatment: upper line = correlation between duodenal damage and (■) GSSG-Rd activity ($r^2 = 0.60$; $F(1,2) = 3.05$; $P < 0.05$). Lower line = correlation between duodenal damage and (□) tGSH concentration ($r^2 = 0.85$; $F(1,2) = 10.99$; $P > 0.05$). Dotted lines delimit confidence interval of duodenal damage: a = control and melatonin groups; b = indomethacin-ethanol-melatonin group; c = indomethacin-ethanol group. Biochemical data were analysed by one way ANOVA followed by Student-Newman-Keul's test. Each symbol represents the mean and vertical lines show s.e.mean. $^{*}P < 0.05$; $^{**}P < 0.01$ vs indomethacin-ethanol group; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$ vs control and melatonin groups.

elicited by melatonin treatment may be either secondary to the pineal hormone-induced decrease in mucosal damage or caused by a direct effect of melatonin on the inflammatory process induced by ethanol administration. In both cases, it contributes to decreased free radical generation and counteracts the alteration of vascular permeability induced by ethanol treatment. An analogous effect of melatonin on PMN infiltration was described by Sewerynek *et al*., 1995b; 1996) in rat liver, following bacterial lipopolysaccharide (LPS) treatment and during hepatic ischaemia-reperfusion in rats. In both these cases, tissue injury is known to be mediated by free radicals generated during the oxidative stress that follows LPS treatment and ischaemia-reperfusion insult.

The antioxidant properties of melatonin have been shown in many experiments in recent years (Hardeland *et al*., 1995; Reiter *et al*., 1995). In *in vitro* experiments, melatonin has proven to be more potent than GSH in inactivating the hydroxyl radical (Tan *et al*., 1993) and twice as potent as vitamin E in scavenging the peroxyl radical (Pieri *et al*., 1994), although this latter feature of melatonin has been questioned (Scaiano, 1995; Marshall *et al*., 1996). Moreover, melatonin scavenges hypochlorous acid at a rate sufficient to protect catalase against inactivation by this molecule, and prevents the oxidation of ox-brain phospholipids in a concentration-dependent manner (Marshall *et al*., 1996). *In vivo* experiments have revealed the protective effect of melatonin against oxidative damage induced by the chemical carcinogen safrole, by the herbicide paraquat and by LPS (Tan *et al*., 1994; Melchiorri *et al*., 1995a; 1996; Seweryneck *et al*., 1995a,b); these agents are known to damage tissues by generating free radicals.

Due to its high lipid (Reiter *et al*., 1994) as well as aqueous solubility (Shida *et al*., 1994), melatonin can easily reach every subcellular compartment and can potentially protect cells from oxidative stress occurring anywhere in the organism (Reiter *et al*., 1995). Moreover, *in vitro* studies (Poeggeler *et al*., 1995) have shown that the pineal hormone acts synergistically with endogenous radical scavengers such as GSH, vitamin E and ascorbate. Interestingly, melatonin exerts potent antioxidant

activities even in the presence of low concentrations of endogenous antioxidants, far below those concentrations that are commonly detected *in vivo*.

Sulphyldryl compounds have been shown to protect the gastric mucosa from ethanol-induced damage both in animals and man (Szelenyi & Brune, 1988; Loguercio *et al*., 1993). GSH is a major endogenous antioxidant in the organism and it is present in high concentrations in the stomach and bowel of both rodents and man (Boyd *et al*., 1979; Hoppenkamps *et al*., 1984; Siegers *et al*., 1984; 1989). The reduction in tGSH concentration elicited by ethanol in the present study deprives the cell of one of its most important antioxidants and impairs the balance between GSSG and GSH concentrations. In contrast to tGSH, GSSG levels remained unchanged following ethanol and indomethacin-induced administration. Thus, either tGSH depletion is not due to GSH conversion to its oxidized form or both tGSH and GSSG are partially lost from the damaged mucosa. Being only a small percentage of tGSH, GSSG could be significantly lost through a leakage from mucosal lesions in the gastroduodenal lumen, thus preventing an increase in GSSG levels.

More difficult to explain is the dramatic decrease in tGSH concetration. The reduction could be due to the oxidation of GSH with the subsequent leakage of GSSG into the gastroduodenal lumen; but this seems unlikely to be the primary cause of tGSH depletion. Alternatively, a block in the synthesis of the tripeptide induced by ethanol-generated free radicals may occur. GSH synthesis is regulated by the amount of cysteine available (Deneke & Fanburg, 1989). Ethanol treatment has been shown to decrease in the level of gastric cysteine by 50% (Loguercio *et al*., 1993). Thus, it is reasonable that oxygen free radicals, generated by ethanol administration, converted gastric and duodenal cysteine to cystine thereby inhibiting the synthesis of GSH. Moreover, the impairment of GSSG-Rd activity which would decrease the rate of conversion of GSSG to GSH, could contribute to GSH depletion. Melatonin protection against ethanol-induced decrease in tGSH concentration, as occurred in the present study, is consistent with previous experiments in which the pineal hormone afforded protection against oxidative stress by restoring the normal GSH concentration in the organism (Melchiorri *et al*., 1995a; 1996; Sewerynek *et al*., 1995a). Also, melatonin administration prevented cataract formation in newborn rats treated with the GSH synthesis inhibitor, buthionine sulphoximine (Abe *et al*., 1994).

The drop in GSSG-Rd activity correlates well with data from previous study by Hirashi *et al*. (1991) in which gastric cell susceptibility to lysis by H_2O_2 was shown to be inversely related to endogenous GSSG-Rd activity. The authors demonstrate that GSSG-Rd is much more potent that endogenous catalase in protecting cultured gastric mucosal cells from oxidative stress. These data reveal the importance of the GSH system as a first line of antioxidant defense in the stomach (Hirashi *et al*., 1991).

The impairment of GSSG-Rd induced by ethanol administration is probably due to the generation of free radicals at a rate that overwhelms the naturally occurring defense mechanisms of the cell (Pigeolet & Remacle, 1991). Melatonin administration completely or partially overcame ethanol-induced changes in GSSG-Rd activity in the gastroduodenal tract. Interestingly, the changes in tGSH concentration as well as the decrease in GSSG-Rd activity in the gastroduodenal tract directly correlate with the level of ethanol-induced gross gastroduodenal damage caused by ethanol administration in both the stomach and duodenum. The same direct correlation was evident when we analysed melatonin protection against ethanol-induced gastroduodenal injury. The administration of melatonin decreased the gastroduodenal morphological damage induced by ethanol treatment with the level of protection being linearly correlated with the action of melatonin on tGSH concentration and GSSG-Rd activity. Indomethacin-ethanol treatment caused a worse morphological and histological picture both in the stomach and duodenum, while influencing only partially the biochemical parameters. Melatonin protected effectively against indomethacin-ethanol-induced morphological and histological changes and PMN accumulation, being less effective in counteracting the biochemical changes induced by the co-administration of indomethacin and ethanol.

Among the most important mechanisms of mucosal protective drugs are increases of endogenous prostaglandins and inhibition of gastric secretion. The cytoprotective action of prostaglandins is well known (Robert *et al*., 1983). It occurs at concentrations far below those needed for the antisecretory effects of these compounds. Indomethacin administration suppresses the activity of cyclo-oxygenase, thus preventing the formation of prostaglandins. It is thus possible that indomethacin administration, by decreasing the endogenous defence mechanisms of the gastroduodenum, increased its vulnerability to ethanol-generated free radicals. Under these conditions, the protective action of melatonin, while still effective on some parameters, is somewhat less obvious when other parameters of damage were considered.

Since aspirin-induced, but not ethanol-induced gastric mucosal damage can be diminished by antisecretory compounds (Pihan *et al*., 1987), the protection conferred by melatonin in our study cannot by due to increase of intragastric pH. Intragastric administration of ethanol increases, by itself, intragrastric pH. The administration of prostaglandins has been shown to reduce the increase in gastric pH elicited by ethanol (Tarnawski *et al*., 1985). However, in our studies, melatonin-ethanol treated animals did not show any significant difference in intragastric pH compared to the ethanol group (data not shown).

On consideration of the large amount of data that have accumulated in recent years related to the antioxidant activity of melatonin, it is likely that the protection afforded by the pineal hormone against ethanol-induced gastroduodenal injury was due, at least in part, to its radical scavenging activity.

References

ABE, M., REITER, R.J., ORHII, P.B. & POEGGELER, B. (1994). Inhibitory effect of melatonin on cataract formation in new born rats: Evidence for an antioxidant role of melatonin. *J. Pineal Res*., **17,** 94–100.

BABIOR, B., KIPNES, R.S. & CURNUTTE, J.T. (1973). Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest*., **52,** 741–744.

BECK, L.V., RIECK, V.D. & DUNCAN, B. (1958). Diurnal variation in mouse and rat liver sulphydryl. *Proc. Soc. Exp. Biol. Med*., **97,** 229–331.

BOYD, S.C., SASAME, H.A. & BOYD, M.R. (1979). High concentrations of glutathione in the glandular stomach: possible implications for carcinogensis. *Science*, **205,** 1010–1012.

DEL MAESTRO, R.F., BJORK, J. & ARFORS, K.E. (1981). Increased microvascular permeability induced by enzymatically generated free radicals. I. In vivo study. *Microvasc. Res*., **22,** 274–277.

DENEKE, S.M. & FANBURG, B.L. (1989). Regulation for cellular glutathione. *Am. J. Physiol*., **257,** L163–L173.

EASTWOOD, G.L. & KIRCHNER, J.P. (1974). Changes in the fine structure of mouse gastric epithelium produced by ethanol and urea. *Gastroeneterology*, **67,** 71–84.

GLASS, G.B.J., SLOMIANY, B.L. & SLOMIANY, A. (1979). Biochemical and pathological derangements of the gastrointestinal tract following acute and chronic ingestion of ethanol. In *Biochemistry and Pharmacology of Ethanol*. Vol. 1. ed. Majchrowicz, E. & Noble, E.P. pp 551–586, New York: Plenum Press.

GRIFFITH, O.W. (1985). Glutathione and glutathione disulphide. In *Methods in Enzymatic Analysis*. Vol. III, ed. Bergmeyer, H.U. pp. 521–531. Weinheim: VCH Verlagsgesellschaft.

GOLDBERG, D.M. & SPOONER, R.J. (1985). Glutathione reductase. In *Methods in Enzymatic Analysis*. Vol. III, ed. Bergmeyer, H.U. pp. 258–265. Weinheim: VCH Verlagsgesellschaft.

HABER, F. & WEISS, J. (1934). The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Ser.*, **A147,** 332–351.

HARDELAND, R., BALGER, I., POEGGELER, B., FURRBERG, B., URIA, H., BEHRMANN, G., WOLF, R., MEYER, T.J. & REITER, R.J. (1995). On the primary functions of melatonin in evolution: mediators of photoperiodic signals in unicell, photooxidation and scavenging of free radicals. *J. Pineal Res.*, **18,** 104–111.

HIRASHI, H., TERANO, A., OTA, S., MUTOCH, H., SUGIMOTO, T., RANDAZI, M. & IVEY, K.J. (1991). Antioxidant defenses of cultured gastric cells against oxygen metabolites: role of GSH redox cycle and endogenous catalase. *Am. J. Physiol.*, **261,** G921–G928.

HOPPENKAMPS, R., THIES, E., YOUNES, M. & SIEGERS, C.P. (1984). Glutathione and GSH-dependent enzymes in the human gastric mucosa. *Klin. Wochenschr.*, **62,** 183–186.

ITOH, M. & GUTH, P.H. (1985). Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology*, **82,** 9–15.

JASKOT, R.H., CHARLET, E.G., GROSE, E.C. & GRADY, M.A. (1983). An automated analysis of glutathione peroxidase, S-transferase and reductase activity in animal tissue. *J. Anal. Toxicol.*, **7,** 86–88.

KATON, R.M. & SMITH, F.H. (1973). Panandoscopy in the early diagnosis of acute upper gastrointestinal bleeding. *Gastroenterology*, **65,** 728–735.

LACY, E.R. & ITO, S. (1982). Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. *Gastroenterology*, **83,** 619–625.

LOGUERCIO, C., TARANTO, D., BENEDUCE, F., DEL VECCHIO BLANCO, C., DE VINCENTIIS, A., NARDI, G. & ROMANO, M. (1993). Glutathione prevents ethanol induced gastric mucosal damage and depletion of sulphydyrl compounds in humans. *Gut*, **34,** 161–165.

LULU, D.J. & DRAGSTEDT, L.R. (1970). Massive bleeding due to acute hemorrhagic gastritis. *Arch. Surg.*, **101,** 550–554.

MARSHALL, K.A., REITER, R.J., POEGGELER, B., ARVOMA, O.I. & HALLIWELL, B. (1996). Evaluation of the anti-oxidant activity of melatonin in vitro. *Free Rad. Biol. Med.*, **21,** 307–315.

MELCHIORRI, D., REITER, R.J., ATTIA, A.M., HARA, M., BURGOS, A. & NISTICÒ, G. (1995a). Potent protective effect of melatonin on in vivo paraquat-induced oxidative damage in rats. *Life Sci.*, **56,** 83–89.

MELCHIORRI, D., REITER, R.J., SEWERYNEK, E., CHEN, L.-D. & NISTICÒ, G. (1995b). Melatonin reduces kainate-induced lipid peroxidation in homogenates of different brain regions. *FASEB J.*, **9,** 1205–1210.

MELCHIORRI, D., REITER, R.J., SEWERYNEK, E., HARA, M., CHEN, L.-D. & NISTICÒ, G. (1996). Paraquat toxicity and oxidative damage. Reduction by melatonin. *Biochem. Pharmacol.*, **51,** 1095–1099.

PARKS, D., BULKLEY, G., GRANGER, D., HAMILTON, S. & MCCORD, J. (1982). Ischemic injury in the cat small intestine: Role of superoxide radicals. *Gastroenterology*, **82,** 9–15.

PARKS, D.A., BURKLEY, G.B. & GRANGER, D.N. (1983). Role of oxygen-derived free radicals in digestive tract diseases. *Surgery*, **94,** 415–422.

PIERI, C., MARRA, M., MORONI, F., RECCHIONI, R. & MARCHESELLI, F. (1994). Melatonin: a peroxyl radical scavenger more potent than vitamin E. *Life Sci.*, **56,** 83–89.

PIGEOLET, E. & REMACLE, J. (1991). Susceptibility of glutathione peroxidase to proteolysis after oxidative alteration by peroxides and hydroxyl radicals. *Free Rad. Biol. Med.*, **11,** 191–195.

PIHAN, G., REGILLO, C. & SZABO, S. (1987). Free radicals and lipid peroxidation in ethanol- or aspirin-induced gastric mucosal injury. *Dig. Dis. Sci.*, **32,** 1395–1401.

POEGGELER, B., REITER, R.J., HARDELAND, R., SEWERYNEK, E., MELCHIORRI, D. & BARLOW-WALDEN, L.-R. (1995). Melatonin, a mediator of electron transfer and repair reactions, acts synergistically with the chain-breaking antioxidants ascorbate, trolox and glutathione. *Neuroendocrinol. Lett.*, **17,** 87–92.

REITER, R.J. (1995). Oxidative processes and antioxidant defense mechanisms in the aging brain. *FASEB J.*, **9,** 526–533.

REITER, R.J., TAN, D.-X., POEGGELER, B., MENDENDEZ-PELAEZ, A., CHEN, L.-D. & SAARELA, S. (1994). Melatonin as a free radical scavenger: implications for aging and age-related diseases. *Ann. New York Acad. Sci.*, **719,** 1–12.

REITER, R.J., MELCHIORRI, D., SEWERYNEK, E., POEGGELER, B., BARLOW-WALDEN, L.R., CHUANG, J.J., ORTIZ, G.G. & ACUNA-CASTROVIEJO, D. (1995). A review of the evidence supporting melatonin's role as an antioxidant. *J. Pineal Res.*, **18,** 1–12.

ROBERT, A., NEZAMIS, E., LANCASTER, C., DAVIS, J.P., FIELD, S.O. & HANCHAR, A.J. (1983). Mild irritants prevent necrosis through 'adaptive cytoprotection' mediated by prostaglandins. *Am. J. Physiol.*, **245,** G113–G121.

SCAIANO, J.C. (1995). Exploratory laser flash photolysis study of free radical reactions and magnetic field effects in melatonin chemistry. *J. Pineal Res.*, **19,** 189–195.

SEWERYNEK, E., ABE, M., REITER, R.J., BARLOW-WALDEN, L.R., CHEN, L.-D., MCCABE, T.J., ROMAN, L.J. & DIAZ-LOPEZ, B. (1995a). Melatonin administration prevents lipopolysaccharide-induced oxidative damage in phenobarbital-treated rats. *J. Cell. Biochem.*, **58,** 346–353.

SEWERYNEK, E., MELCHIORRI, D., REITER, J.R., ORTIZ, G.G. & LEWINSKI, A. (1995b). Lipopolysaccharide-induced hepatotoxicity is inhibited by the antioxidant melatonin. *Eur. J. Pharmacol.*, **293,** 327–334.

SEWERYNEK, E., MELCHIORRI, D., CHEN, L.-D. & REITER, R.J. (1995c). Melatonin reduces both basal and bacterial lipopolysaccharide-induced lipid peroxidation in vitro. *Free Rad. Biol. Med.*, **19,** 903–909.

SEWERYNEK, E., REITER, R.J., MELCHIORRI, D., ORTIZ, G.G. & LEWINSKI, A. (1996). Oxidative damage in the liver induced by ischemia-reperfusion: Protection by melatonin. *Hepato-Gastroenterology*, **43,** 898–905.

SHAW, S., HERBERT, V., COLMAN, N. & JAYATILLEKE, E. (1990). Effect of ethanol-generated free radicals on gastric intrinsic factor and glutathione. *Alcohol*, **7,** 153–157.

SHIDA, C.S., CASTRUCCI, A.M.L. & LAMY-FREUND, M.T. (1994). High melatonin solubility in aqueous medium. *J. Pineal Res.*, **16,** 198–201.

SIEGERS, C.P., BOSE-YOUNES, H., THIES, E., HOPPENKAMPS, R. & YOUNES, M. (1984). Glutathione and GSH-dependent enzymes in the tumorous and non tumorous mucosa of the human colon and rectum. *J. Cancer Res. Clin. Oncol.*, **107,** 238–241.

SIEGERS, C.P., BARTELS, L. & RIEMAN, D. (1989). Effects of fasting and glutathione depletors on the GSH-dependent enzyme system in the gastrointestinal mucosa of rats. *Pharmacology*, **38,** 121–128.

SZABO, S. (1981). Sulphydryl compounds may mediate gastric cytoprotection. *Science*, **214,** 220–222.

SZABO, S. (1987). Mechanisms of mucosal injury in the stomach and duodenum: time-sequence analysis of morphologic, functional, biochemical and histochemical studies. *Scand J. Gastroenterol.* Suppl., **127,** 21–28.

SZELENYI & BRUNE, K. (1988). Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. *Dig. Dis. Sci.*, **33,** 865–871.

TAN, D.-X., CHEN, L.-D., POEGGELER, B., MANCHESTER, L.C. & REITER, R.J. (1993). Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocrine J.*, **1,** 57–60.

TAN, D.-X., REITER, R.J., CHEN, L.-D., POEGGELER, B., MANCHESTER, L.C. & BARLOW-WALDEN, L. (1994). Both physiological and pharmacological levels of melatonin reduce DNA adduct formation induced by the chemical carcinogen safrole. *Carcinogenesis*, **15,** 215–218.

TARNAWSKI, E., STACHURA, J., IVEY, K., MACH, T., BOGDAL, J. & KLIMCZYK, B. (1981). Ethanol-induced duodenal lesions in man. Protective effect of prostaglandin. *Prostagladins*, **21**(suppl), 147–153.

TARNAWSKI, A., HOLLANDER, D., STACHURA, J., KRAUSE, W.J. & GERGELY, H. (1985). Prostaglandin protection of the gastric mucosa against alcohol injury - A dynamic time-related process. *Gastroenterology*, **88,** 334–352.

WINER, B.J. (1971). *Statistical Principles in Experimental Design*. ed. McGraw, H. II edition, U.S.A: McGraw-Hill Book Company.

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