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EFFECTS OF THE PHENOLIC ANTIOXIDANT 2,5-DI-TERT-BUTYL-4-HYDROXYANISOLE (DTBHA) ON GASTRIC MUCOSAL DAMAGE AND LIPID PEROXIDATION STATUS IN COLD/RESTRAINT STRESSED RATS

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ABSTRACT

Gastric ulcer is a multifactoral disease with pathogenesis still not completely understood. There is increasing evidence that stressors of physical and chemical origin can induce gastric mucosal injury through enhanced production of oxygen species and oxidative stress, but the role of antioxidants in gastric protection is controversial. The present study aimed the evaluating the effect of the phenolic antioxidant DTBHA (2,5, 5,0, and 10,0mg/kg, p.o.) afainst gastric mucosal damage induced by cold/restraint stress (CRS: immobilization for 4 hours at 4°C) in rats. Morphometrical and histomorphometrical studies have been carried out. Mean ulcer area has been calculated. Lipid peroxidation in plasma, red blood cells (RBC) and gastric mucosa was estimated. The results showed that CRS induced mucosal lesions varying in number and size . Elevated plasma levels of malondialdehyde (MDA) and uric acid, almost 6- and 3-folds respectively, were found. MDA was significantly increased in RBC and slightly increased in gastric mucosa. Decreased levels of reduced glutathione (GSH) in RBC and a trend to increase in gastric GSH was found. The pretreatment with DTBHA in a dose of 5 and 10 mg/kg significantly decreased the mean ulcer area by 63,2% and 90,4% respectively. Histomorphological study revealed almost normal gastric appearance in these cases. Significant decrease in stress-enhanced plasma, RBC and gastric mucosa MDA was found in DTBHA 5,0 mg/kg pretreated animals. Uric acid and glutathione levels remained slightly changed after DTBHA. The present findings allowed suggestion that the protective effect of DTBHA against acute CRS-induced gastric mucosal damage depends, at least partially, on the inhibition of lipid peroxidation.

Key words: CRS, RBC, gastric mucosa, DTBHA, oxidative stress, lipid peroxidation

INTRODUCTION

Peptic ulcer is a multifactorial disease with pathogenesis still not completely understood. Recently, there is increasing evidence that stressors of physical and chemical origin can induce gastric mucosal injury through enhanced production of oxygen species and oxidative stress. Oxidative injury is recognized to play important role in gastric ulceration caused by indomethacin (30), ethanol (5,24), pylorus-ligation (25), *Helicobacter pylori* (9), or ischemia and hypotensive shock (8,24). Mucosal ischemia and subsequent local release of tissue-damaging oxygen-derived free radicals is thought to be the major causative factor implicated in the pathogenesis of stress ulceration (6,7).

Although numerous agents with well-proven antioxidant activity such as rutin (20), quercetin (17), sylimarin (1), butylated hydroxytoluene (BHT) (18), vitamin E and selenium (2) have been shown to protect gastric mucosa against various ulcerogenic stimuli, the role of antioxidants in gastric protection remains controversial.

In the present work we have studied the effects of 2,5-di-tert-butyl-4-hydroxyanisole (DTBHA), a derivative of the phenolic compound butylated hydroxyanisole (BHA), widely used for food preservation on gastric mucosal damage caused by cold/restraint stress in rats and lipid peroxidation status in gastric mucosa, blood plasma and red blood cells (RBC). Oxidative stress has been evaluated by measuring thiobarbituric acid reactive substances as end product of lipid peroxidation as well as by uric acid and glutathione levels as markers of the antioxidant defense.

MATERIAL AND METHODS

A. Ulcer induction

Male Wistar rats weighting 240-270g were used. The animals were housed under controlled environmental conditions and on standard food diet. They were deprived of food

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24h before the experiment at a free access to water. DTBHA (2,5, 5,0 and 10,0mg/kg) was administered per sondam in a volume of 0,2ml/100g 60 min before ulcer induction. Control animals were given water instead DTBHA in the same test schedule and under the same conditions. Each experimental group consisted of at least 8 animals. Gastric mucosal damage was evoked by cold/restraint stress (CRS). The animals were placed in individual restraint plastic boxes 20x10x10cm and exposed to temperature 4°C±1°C for 4h. The experimental procedure was approved by Home Office for Care and Use of Laboratory Animals and performed respecting humanity of animal experiments.

After the stress period animals were sacrificed by rapid decapitation and exsanguination. The stomach was removed immediately, opened along the greater curvature, gently washed in physiological salt solution, spread over a pad and observed macroscopically for appearance of mucosal lesions. The length of each lesion was measured. In case of petechia, 5 of them were considered as 1mm lesion. Mean ulcer area (mm^2) was calculated.

Pieces of the stomach were immersed into 8% formalin solution, embedded in paraffin and stained with hematoxilin-eosin. Histomorphological examination was performed under light microscopy and documented by Jena Lumar microphotocamera.

C. Biochemical examinations

Blood was taken from the femoral vein and heparinized. Plasma was separated by centrifugation at 2000rpm for 10 min and aliquots were stored at -80°C until analysis. Physiologic saline solution (pH=7,4) was used for washing the RBC pellet triply. To the pellet of washed RBC equal volume of cold distilled water was added vortexed and frozen at -20°C for 30min. Immediately after thawing the plasma and hemolysates were used for analysis.

Stomach tissue was rinsed with ice-cold physiological saline solution and homogenized in 50mM phosphate buffer (pH=7,40), containing 0,1mM EDTA (Potter-Elveheim Teflon - glass homogenizer). Membrane lipid peroxidation was monitored by measuring malondialdehyde (MDA) - an end product of fatty acid peroxidation. MDA was assayed by its thiobarbituric acid (TBA) reactivity in plasma, RBC and stomach tissue homogenates using the method of Porter *et al.* (21). MDA values were determined using the extinction coefficient of the TBA-MDA complex at 532nm = 1,56 x 103 per cm per molar solution. Uric acid was measured in plasma by the method of Bergmeyer (1981) (4). Reduced (GSH) and oxidized (GSSG) glutathione content were assayed according to the method of Hissin and Hilf (1976) (15), using o-phthaldialdehyde as fluorescent agent. Standard solutions of GSH and GSSG were applied to calculate the glutathione content. Protein content in stomach tissue homogenates was measured according to Biurett method using Biotest La Chema.

D. Statistical analysis

Data were analysed statistically by Student's *t*-test using GraphPad Prism. A value of $p<0,05$ was considered statistically significant.

RESULTS AND DISCUSSION

A. Effect of DTBHA on CRS-induced gastric mucosal damage

The macroscopic observation showed that CRS induces gastric mucosal lesions of varying number and size. The area of involvement was confined to the glandular part of the stomach. There was evidence of intraluminal bleeding. The lesion bed was filled with a brown-colored haematin material slightly fixed to the liable tissue. The mean ulcer area was 13,60 ±2,30mm^2 (Table 1). The pretreatment with DTBHA of 2,5mg/kg did not change significantly the ulcer area, while the pretreatment with DTBHA of 5,0 and 10,0mg/kg resulted in a marked decrease in ulcer area by 63,20% ($p<0,05$) and 90,40% ($p<0,001$), respectively (Table 1).

Table 1. Effect of DTBHA on CRS-induced gastric mucosal damage in rats

Experimental group	Mean ulcer area (mm^2)	Alteration (%)
Control (n=10)	13,6 ±2,3	100
DTBHA 2.5 mg/kg (n=8)	16,4 ±4,6	120,6
DTBHA 5.0 mg/kg (n=8)	5,0 ±2,2*	46,8
DTBHA 10.0 mg/kg (n=8)	1.3 ±0,6**	9,6

* $p<0.05$; ** $p<0.001$

B. Histopathological study

The histopathological study showed that the CRS-induced lesions were manifested as acute erosive defects more often superficial than implicating the total thickness of the mucosa. In the lesion area the superficial epithelial layer and fosses were absent. The glandular part of the wall was partially affected. Lesions were filled with blood coagulates and haematin material. The cytoplasm of the superficial epithelium in adjacent areas showed signs of mucous dysplasia. In the group pretreated with DTBHA the gastric mucosal appearance was almost normal. In some cases only bending of the superficial epithelium and focal desquamation was found.

C. Biochemical examination

In the CRS group gastric mucosal MDA tended to increase (9,94%). Compared to it, after DTBHA 2,5, 5,0 and 10,0mg/kg MDA level was decreased by 29,92%, 18,92% and 18,27%, respectively (Table 2). In all experimental groups pretreated with DTBHA a significant decrease in plasma MDA levels by 40,28%, 52,96% and 23,10% in comparison with CRS was found. Compared to controls plasma MDA remained significantly enhanced.

Significant increase in mucosal uric acid levels, towards the controls, for all groups pretreated with DTBHA was detected.

Table 2. Effect of DTBHA on biochemical parameters for evaluation of oxidative stress in blood plasma, RBC and stomach tissue homogenate

Experimental group	Blood plasma		RBC		Stomach tissue homogenate	
	MDA [nmol/ml]	UA [mg%]	MDA [nmol/ml lysate]	GSH [ug/ml lysate]	MDA [nmol/mg protein]	GSH [ug/mg protein]
Control (n=10)	0,6 ±0,06	1,0 ±0,07	6,7 ±1,1	161,7 ±12,4	9,7 ±0,3	5,6 ±0,3
CRS (n=10)	3,5 ±0,1 ^^^	2,7 ±0,3 ^^^	9,5 ±0,7	108,4 ±28,4	10,73 ±0,7	8,6 ±0,4 ^^^
DTBHA +CRS 2.5 mg/kg (n=8)	2,1 ±0,1 *** ^^^	3,2 ±0,3 ^^^	8,5 ±1,1	100,6 ±37,5	7,5 ±0,6 ** ^^	8,1 ±1,1 ^
DTBHA +CRS 5.0 mg/kg (n=8)	1,6 ±0,2 *** ^^^	1,7 ±0,2 * ^^	7,1 ±0,3 **	128,5 ±15.6	8,7 ±0,5 *	5,9 ±0,5
DTBHA +CRS 10.0 mg/kg (n=8)	2,7 ±0,2 ** ^^^	2,1 ±0,8	8,3 ±0,5	140,0 ±28,0	8,8 ±0,6	7,8 ±0,5 ^^

^p< 0.05 vs. control; ^^ p< 0.01 vs. control; ^^^ p< 0.001 vs. control
*p<0.05; ** p<0.01; *** p< 0.001 vs CRS-group

MDA content in RBC of CRS-animals was elevated by 23,10%. In DTBHA-pretreated groups MDA was significantly decreased by 10,80%, 25,16% and 12,68% for DTBHA 2,5, 5,0 and 10,0mg/kg, respectively. CRS provoked reduction in RBC-GSH 32,97%. Complete restoration after DTBHA 10,0mg/kg was found for GSH (+29,13%).

It has been recently shown that stress-evoked gastric mucosal lesions are closely related to extensive lipid peroxidation in gastric tissue evidenced by accumulation of MDA and depletion of glutathione level (23,29,6,7), although conflicting results in this aspect are also reported. Increased gastric TBARS levels and decreased nonprotein sulfhydryl content after 60 min CRS were found by numerous authors (26,27). Unchanged TBARS and GSH levels in stomach tissue accompanied by significantly increased gamma-glutamylcysteine synthetase activity (responsible for GSH synthesis) and unchanged gamma-glutamyltranspeptidase activity (responsible for GSH breakdown) was determined after water-immersion stress (3). Manifested CRS-induced gastric ulceration with elevated gastric TBARS levels but unchanged GSH content was reported by other autors (27, 29).

In the present work we have studied stress-induced gastric ulceration using CRS as one of the most widely used and reliable stress models (19). We found that the single exposure to CRS in rats induced severe gastric mucosal damage and DTBHA markedly reduced its morphometrical and histomorphological manifestations. Slight changes in gastric MDA were found on the 4th hour after CRS-induction. At the same time the values of GSH were markedly enhanced. As MDA represents a final product of the lipid peroxidation process it is reasonable to suppose a time-course of the oxidative stress-induced pathological chain in which the accumulation of the lipid peroxidation products evidenced late. Decrease in gastric mucosal MDA values and keeping high levels of gastric mucosal GSH could be interpreted as favorable consequences of DTBHA pretreatment.

It has been recently reported that increased plasma lipid peroxide levels (estimated as MDA) also support the role of lipid peroxidation in ulcerogenesis, since plasma lipid peroxide levels reflect a change in the antioxidant/oxidant state of different tissues (26,29). We found that the exposition to 4-hour CRS dramatically enhanced plasma MDA suggesting lipid peroxidation and oxidative stress development. Significant raise in plasma uric acid levels as a major plasma antioxidant (13,28) has also been found out.

RBC-MDA values were markedly increased in the stressed animals, while RBC-GSH was significantly decreased. Based on of these findings it could be supposed that plasma and RBC are either more susceptible to oxidative stress compared to the gastric mucosa, where MDA and GSH changed in different manner, or in this case the oxidative stress is evidenced earlier. It appears that in gastric mucosa the lipid peroxidation has started independently of the changes in GSH levels (3).

The tested compound DTBHA was found to prevent the CRS-induced raise in plasma- and RBC-MDA effectively, to maintain high plasma uric acid levels and to restore stress-reduced levels of RBC-GSH. Taking into consideration the observed changes in the parameters under investigation, well pronounced in RBC and more discrete in gastric mucosa, it could be supposed that the protective effects of DTBHA against acute gastric ulceration which is one of the stress manifestations is related to its antioxidant capacity. The interference with the lipid peroxidation is one of the possible ways to explain the protective effect of DTBHA against mucosal lesions. It should be noted, that the enhanced contractility of the gastric wall accompanied by reduced gastric mucosal blood flow represents one of the major factors of the stress ulceration. Moreover, the vascular dysfunction is one of the events occurring early in the overall pattern of the development of gastric mucosal injury (14). Some calcium antagonists have been found to reduce stress-induced gastric motor hyperactivity and stress-gastric mucosal lesions, confirming the possibility gastric ulceration to be controlled by calcium modulators. It has been recently shown that besides their well-known antioxidant activity a series of phenols that are structurally related to BHA, including DTBHA, have antispasmolytic and spasmolytic effects in rat ileum longitudinal muscle (10,11,12,22) possibly due to their non specific calcium antagonistic activity. It can be supposed that the antispasmolytic effect of DTBHA on both vascular and non-vascular smooth muscle tissues is one of the important factors protecting gastric mucosa against stress ulceration. Preserving the defensive role of the gastric microcirculation thus preventing ischemia/reperfusion phenomenon, which triggers intracellular Ca^{2+}-overload and following lipid peroxidation, DTBHA could interfere with some of the most important pathogenetic factors of the stress-induced gastric ulceration. Similarly to a wide range of compounds with antioxidant activity, including phenols (16) it could be supposed that DTBHA possesses a lipoxygenase inhibiting activity. As leukotrienes have pathogenetic role in gastric ulceration due to potent alteration of the mucosal microvasculature, an inhibition of leukotriene synthesis could be interpreted as an additional mechanism by which DTBHA protects gastric mucosa against stress-lesion formation.

CONCLUSION

DTBHA protects gastric mucosa against acute stress-induced gastric mucosal lesions. Even the precise mechanism remains unclear the results of the present study allow to suggest that the observed gastroprotection at least partly could be due to inhibition of lipid peroxidation and increased antioxidant defense.

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