Attenuation of cold restraint stress-induced gastric lesions by an olive leaf extract

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Abstract. Olive leaf extract (OLE) possesses, among other, antioxidative properties, but whether it influences gastroprotection against stress-induced gastric lesions remains unknown. In this study we investigated the protective effect of OLE, a natural antioxidant, on gastric mucosal damage induced by cold restraint stress (CRS) in rats. Three different doses of commercial OLE EFLA® 943 were applied intragastrically (i.g.) 30 min prior to stress induction. Macroscopic gastric lesions were evaluated and ulcer index (UI) was calculated. Histological evidence of gastric mucosal lesions was also obtained. Concentration of malondialdehyde (MDA) as an index of lipid peroxidation, and catalase (CAT) and superoxide dismutase (SOD) activities were determined in gastric mucosa. The effects of applied OLE on gastric mucosal lesions, lipid peroxidation and antioxidative enzymes activity were compared with effects of i.g. pretreatment of reference drug, ranitidine. CRS caused severe gastric lesions in all non-pretreated animals, and this finding was confirmed histologicaly. Pretreatment with OLE (40, 80 and 120 $mg \cdot kg^{-1}$), as well as with ranitidine (50 $mg \cdot kg^{-1}$), significantly ($p < 0.001$) attenuated stress-induced gastric lesions. Treatment with 80 $mg \cdot kg^{-1}$ of OLE was the most effective in prevention of rise in gastric MDA level and decrease in CAT and SOD activity. The results obtained indicate that OLE possesses gastroprotective activity against CRS-induced gastric lesions in rats, possibly related to its antioxidative properties.

Key words: Olive leaf — Gastroprotection — Cold restraint stress — Rats

Introduction

Olive (*Olea europaea* L.) leaf has been traditionally used for centuries to prevent and treat different diseases. It is used to enhance the immune system, in heart disease and as an antimicrobial agent. Folk medicine uses also include hypertonia, arteriosclerosis, rheumatism, gout, diabetes mellitus, and fever (PDR for Herbal Medicine 2000), and the most known feature of olive leaf is cardioprotection. Experimental animal studies on different total olive leaf extract (OLE) or their constituents have demonstrated hypoglycemic (Gonzales et al. 1992; Al-Azzawie and Alhamdani 2006), hypotensive (Khayyal et al. 2002; Scheffler et al. 2008), antiarrhythmic (Somova et al. 2004), anti-atherosclerotic (Wang et al. 2008), and vasodilator effects (Zarzuelo et al. 1991). Antimicrobial (Bisignano et al. 1999; Furneri et al. 2002; Markin et al. 2003; Pereira et al. 2007), antiviral (Lee-Huang et al. 2003; Micol et al. 2005), anti-tumor (Hamdi and Castellon 2005; Abaza et al. 2007) and anti-inflammatory activity (Pieroni et al. 1996) were also reported. Moreover, antihypertensive and cholesterol-lowering actions of OLE EFLA®943 were confirmed in a clinical study (Perrinjaquet-Moccetti et al. 2008).

The beneficial properties of olive leaf are further enhanced by the good bioavailability of its polyphenolic constituents, the same as in olive oil, which are readily absorbed through

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the gastrointestinal tract, resulting in significant levels in the circulation (Visioli et al. 2000; Vissers et al. 2002).

Despite a number of papers published on different effects of olive leaf and its constituents, none of them has focused on influence on gastric defense mechanisms and gastroprotective activity of total OLE. However, our recent results indicated that it has beneficial effect on ethanol-induced gastric lesions in rats (Dekanski et al. 2009). Furthermore, it was reported that olive oil, thanks to its minor components, improved antioxidant defense systems in rat stomach (Odabasoglu et al. 2008).

The main constituent of the olive leaves is oleuropeine, one of iridoide monoterpenes, which is thought to be responsible for pharmacological effects. Furthermore, the olive leaves contain triterpenes (oleanolic and maslinic acid), flavonoides (luteolin, apigenine, rutin, ...), and chalcones (olivin, olivin-diglucoside) (PDR for Herbal Medicines 2000; Meirinhos et al. 2005; Pereira et al. 2007). It is its chemical content that makes olive leaf one of the most potent natural antioxidants. Oleuropein has high antioxidant activity *in vitro*, comparable to a hydrosoluble analog of tocopherol (Speroni et al. 1998), as do other constituents of olive leaf (Briante et al. 2002). It was shown that total OLE had antioxidant activity higher than vitamin C and vitamin E, due to the synergy between flavonoids, oleuropeosides and substituted phenols (Benavente-Garcia et al. 2000).

Stress has been shown to be associated with altered homeostasis that may lead to oxidant-antioxidant imbalance. It is well known that the pathogenesis of stress-induced gastric lesions includes the generation of reactive oxygen species (ROS) that seem to play an important role, namely due to generation of lipid peroxides, accompanied by impairment of antioxidative enzyme activity of cells (Das et al. 1997; Kwiecien et al. 2002). Administration of antioxidants such as reduced glutathione or sodium benzoate prior to stress, causes significant decrease in ulcer index (UI) and lipid peroxidation, suggesting the involvement of ROS in cold restraint stress (CRS)-induced gastric ulceration (Das and Banerjee 1993). Therefore, treatment with a potent antioxidant, as total OLE is, could decrease stress-induced gastric mucosal damage. To test this hypothesis, we investigated the protective antioxidative effect of OLE on gastric mucosal damage induced by CRS in rats.

Materials and Methods

Materials

Standardized dry OLE EFLA®943 was purchased from Frutarom Switzerland Ltd. (Wädenswil, Switzerland). The extract was manufactured from the dried leaves of *Olea europaea* L., applying an ethanol (80% m/m) extraction procedure. After a patented filtration process (EFLA®HyperPure) the crude extract was dried. Previously reported quantitative analysis of total phenols, flavonoids and tannins content of OLE (Dekanski et al. 2009) revealed that extract used had high oleuropein content (19.8%), total flavonoids (0.29%), including caffeic acid, luteolin-7-O-glycoside, apigenine-7-O-glycoside and quercetin, as well as tannins (0.52%). Ranitidine tablets were obtained from Galenika a.d. (Belgrade, Serbia). Hydrogen peroxide and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). All other reagents used in biochemical analysis were obtained from Merck (Darmstadt, Germany).

Animals

Male Wistar rats ($n = 36$) from Biomedical Research Center, R&D Institute, Galenika a.d. (Belgrade, Serbia), weighing 180–220 g were used in this study. Rats were housed 3 in a cage under constant environmental conditions (20–24°C; 12 h light-dark cycle), and were given *ad libitum* access to standard pelleted food and water. This study was approved by the Ethical Committee, Medical School, University of Belgrade, and run in accordance to the statements of European Union regarding handling of experimental animals (86/609/EEC).

Gastric lesions induction and evaluation

Before the experiment, the animals were randomly divided into 6 groups (6 rats in each group), they were placed in individual metabolic cages, and they were fasted for 24 h, but had free access to water. The first, control, non-ulcerated group received distilled water intragastricaly (i.g.) using metal tube for gavage and it was the group of normal, healthy animals without any pretreatment or stress induction. The second group received distilled water i.g. 30 min prior to stress induction. We applied three different doses of OLE on the next three groups, and finally, the last group (positive control) received i.g. 50 $mg \cdot kg^{-1}$ of ranitidine, H_2 receptor antagonist, as a reference drug. The dose used in this *in vivo* experiments was based on the average consumption of olive drupes and olive oil (Quaranta and Rotundo 2000) in the Mediterranean area. We expressed total polyphenol consumption from olive drupes or olive oil in Mediterranean diet as the molecular equivalent of oleuropein and its metabolites, calculated to be approximately 100 mg daily. For the extrapolation of the dosage from humans to rats, we used the metabolic body size or food intake rather than body weight as a criterion (Rucker and Storms 2002; Rucker 2007). The estimated quantity of oleuropein expressed per unit of human diet was 0.2 $mg \cdot kg^{-1}$ of dry food (Andreadou et al. 2006). For rats, this consumption corresponded to a dose of 8 mg/kg of oleuropein. It was reported that in

OLE investigated, oleuropein content was 20%. Hence, 40 $mg \cdot kg^{-1}$ of OLE was administered. Higher doses of 80 and 120 $mg \cdot kg^{-1}$ were also given to test for a dose response. Both OLE and ranitidine were suspended in distilled water before administration. To induce cold-restraint stress, the rats were immobilized in individual restraint boxes without possibility of visual contact (Popović et al. 1997) and subjected to cold (4 ± 1°C) stress for 3.5 h. This regimen of cold-restraint stress has been reported to produce gastric ulcers reliably in food-deprived rats (Senay and Levine 1967; Das et al. 1993).

At the end of this period, animals were sacrificed under the ether anesthesia, abdomen was opened by the midline incision, the stomach was removed, opened along the greater curvature, rinsed gently with water and pinned open for macroscopic examination and for photo-documentation by digital camera (Hewlett Packard PhotoSmart R507). The number and severity of gastric lesions were evaluated according to the following rating scale (Buyukcoskun et al. 2007): 0 – no lesion, 1 – mucosal edema and petechiae, 2 – from 1 to 5 small lesions (1–2 mm), 3 – more than 5 small lesions or 1 intermediate lesion (3–4 mm), 4 – 2 or more intermediate lesions or 1 gross lesion (greater than 4 mm) and 5 – perforated ulcers. The sum of the total scores divided by the number of animals in group was expressed as the UI ± SD (standard deviation). Percent of inhibition in UI in relation to the non-pretreated CRS group were estimated from formula:

$$\% \text{ inhibition} = [1 - (\text{UI pretreatment} / \text{UI non-pretreatment})] \times 100$$

Histopathological evaluation

Histological evidence of gastric mucosal lesions in all experimental groups was also obtained. Gastric tissue samples were fixed in 10% buffered formalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Sections of 5 μm thicknesses were cut by rotatory microtome, stained with haematoxylin and eosin, and examined microscopically for histopathological changes using microscope with digital camera (Olympus BX41, Japan) and Olympus DP-SOFT 5.0 programme for photo-documentation.

Biochemical examination of gastric mucosa

The mucosal tissue from each animal was scraped from the stomach with a blunt knife and the tissue was weighed, transferred to the ice-cooled test tube and homogenized by Ultra-Turrax T25, (Janke & Kunkel Gmbh& Co., IKA®-Labortechnik, Staufen, Germany) in 20 mmol/l Tris buffer, pH 7.4, containing 5 mmol butylated hydroxytoluene to prevent new lipid peroxidation that can occur during the homogenization. The homogenate was then centrifuged at 12,000 rpm at 4°C, (Megafuge 2.0.R, Heraeus, Germany) for 10 min. Supernatant was aliquoted and stored at –70°C until determination of total protein, catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA).

The protein content of the tissue samples was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Lipid peroxidation of gastric mucosa was determined spectrophotometrically (UV-Vis spectrophotometer HP 8453, Agilent Technologies, Santa Clara, CA) at 533 nm and MDA concentration was quantified by using the molar extinction coefficient, 1.56×10^5 $mol^{-1} \cdot cm^{-1}$ (Buege and Aust 1978).

Activity of CAT in gastric mucosa was determined according to the procedure of Goth (1991) by following the absorbance of hydrogen peroxide at 230 nm and pH 7.0.

SOD activity in the gastric mucosa was determined by measuring the inhibition of autooxidation of epinephrine at pH 10.2 at 30°C by the method of Misra and Fridovich (1972). One unit of SOD activity represents the amount of SOD necessary to cause 50% inhibition of adrenaline autooxidation.

Statistical analysis

All results are expressed as means ± SD. Statistical analysis was done using *t*-test, and *p* values less than 0.05 were considered as significant.

Results

Effect of OLE suspension on gastric lesions induced by CRS

Cold restraint stress produced visible gastric lesions in all non-pretreated animals. They were located mostly in the corpus. No visible lesions developed in the nonsecretory part of the rat stomach which is well known response to CRS. Moreover, after opening, haemorrhagical content was found in stomach lumens. Following 3.5 h of cold-restraint stress, the average ulcer score in non pretreated group was very high (4.3 ± 0.8). All administered doses of OLE (40, 80 and 120 $mg \cdot kg^{-1}$) significantly prevented the gastric mucosal lesions induced by cold-restraint stress. Percent of inhibition in UI was 49%, 70%, and 63%, respectively. The gastroprotective effect of OLE was similar to that achieved by the pretreatment with H_2 receptor antagonist, ranitidine (50 $mg \cdot kg^{-1}$), which caused 70% inhibition of UI (Fig. 1). No visible sign of ulceration was observed in animals in non-ulcerated group.

Effect of OLE pretreatment on histopathological changes

Histological examination of gastric mucosa showed stress induced extensive damage of the surface epithelium and

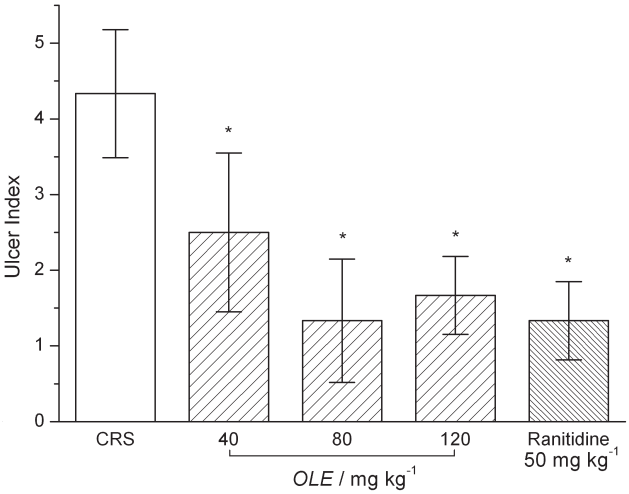

Figure 1. Effect of intragastric pretreatment with olive leaf extract (OLE) applied in graded doses ranging from 40 mg·kg^{-1} up to 120 mg·kg^{-1} and ranitidine (50 mg·kg^{-1}) on the ulcer index induced by CRS. Asterisk indicates statistical significance in ulcer index ($p < 0.001$), as compared to the control value.

lamina propria, with lesions extending up to submucosa in some cases. Various histopathological changes including congestion, haemorrhage, submucosal edema, necrosis, inflammatory changes, erosions and ulcers were seen (Fig. 2A). All these changes were significantly less expressed in rats pretreated with both OLE (Fig. 2B) and ranitidine. Fig. 2C shows normal gastric mucosa.

Effect of OLE pretreatment on lipid peroxidation and antioxidative enzymes activity

Cold restraint stress significantly increased lipid peroxidation in gastric mucosa, evaluated as MDA formation (445.4 ± 43.6 vs. 287.1 ± 15.6 in control; ($p < 0.05$)). MDA was reduced significantly by pretreatment with 80 and 120 mg·kg^{-1} of OLE (23% and 16%, respectively). Administration of 50 mg·kg^{-1} and ranitidine prior to CRS exposure also restricted rise in MDA concentration, but these effects were not statistically significant. Fig. 3 shows MDA concentration in normal gastric mucosa and in gastric mucosa of non-pretreated rats and rats pretreated with OLE and ranitidine.

As shown in Fig. 4, SOD activity averaged 35.5 ± 2.3 U (mg prot)$^{-1}$ in intact gastric mucosa. Following exposure of rats to CRS, a significant decrease of SOD activity to the value of 30.3 ± 0.9 U (mg prot)$^{-1}$ was observed. Both OLE and ranitidine administration reduced the fall in SOD activity, but only effects of 80 and 120 mg·kg^{-1} of OLE were statistically significant.

Catalase activity in gastric mucosa, also, significantly decreased after CRS (17.7 ± 5.2 U (mg prot)$^{-1}$ in control group vs. 9.2 ± 3.5 U (mg prot)$^{-1}$ in CRS group). Pretreatment of

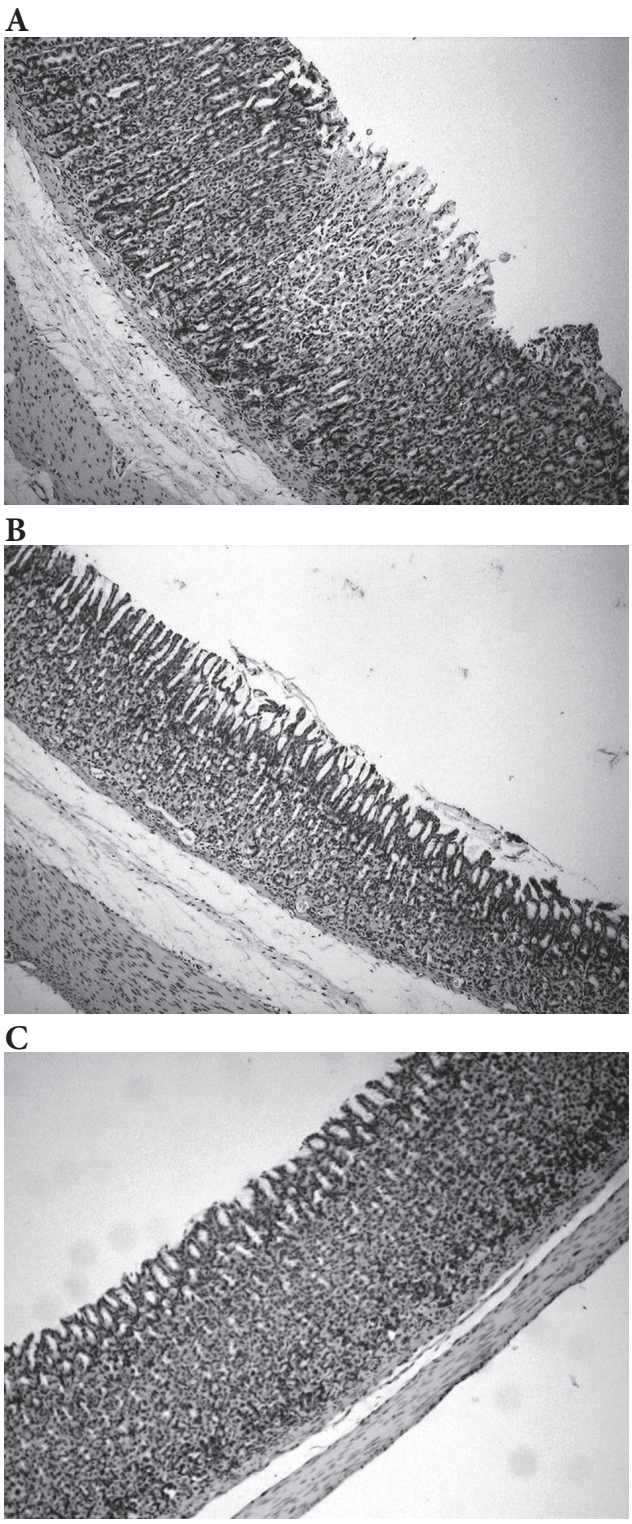

Figure 2. Examples of histopathological changes in stomach of CRS exposed rats. **A.** Deep erosion and massive extravasation of erytrocytes, submucosal edema and cell infiltration in non-pretreated rat. **B.** Surface epithelium damage and submucosal edema without deep erosions and bleeding, in olive leaf extract pretreated rat. **C.** Stomach from control, non-ulcerated rat.

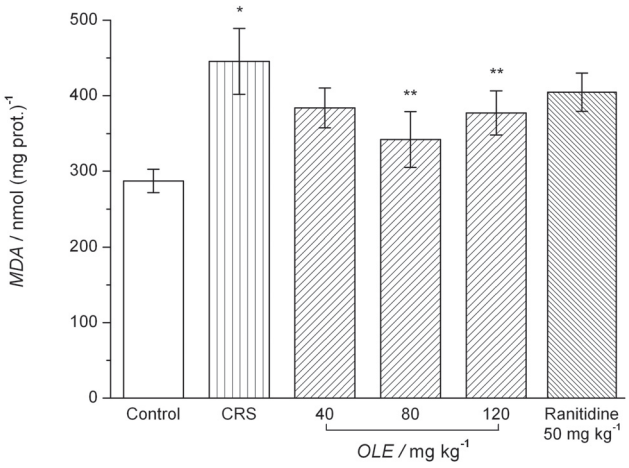

Figure 3. Effect of intragastric pretreatment with olive leaf extract (OLE) applied in graded doses ranging from 40 mg·kg^{-1} up to120 mg·kg^{-1} and ranitidine (50 mg·kg^{-1}) on the malondyaldehyde (MDA) concentration (nmol·mg prot^{-1}) in gastric mucosa. * indicates statistical significance ($p < 0.05$) of difference in MDA concentrations in non-pretreated rats exposed to CRS as compared to the control animals. ** indicates statistical significance ($p < 0.05$) of difference in MDA concentrations in pretreated rats as compared to the CRS exposed rats without pretreatment.

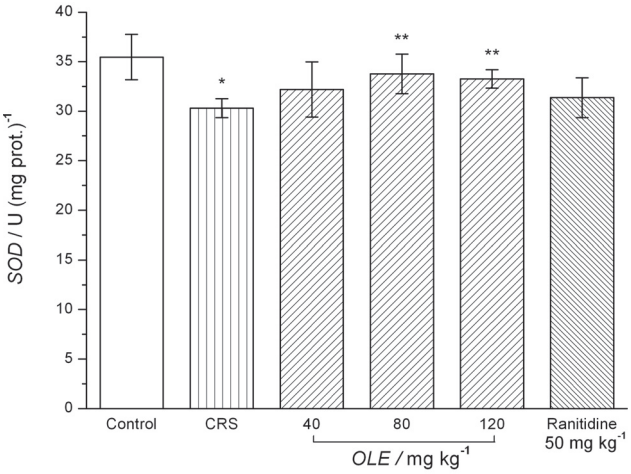

Figure 4. Effect of intragastric pretreatment with olive leaf extract (OLE) applied in graded doses ranging from 40 mg·kg^{-1} up to120 mg·kg^{-1} and ranitidine (50 mg·kg^{-1}) on the SOD activity (U (mg prot)$^{-1}$) in gastric mucosa. * indicates statistical significance ($p < 0.05$) of difference in SOD activity in non-pretreated rats exposed to CRS as compared to the control animals. ** indicates statistical significance ($p < 0.05$) of difference in superoxide dismutase (SOD) activity in pretreated rats as compared to the CRS exposed rats without pretreatment.

all three doses of OLE significantly reduced the fall of CAT activity, while the effect of ranitidine was not statistically significant (Fig. 5).

Discussion

There is an increasing interest in medicinal plant extracts, the greatest value of which may be due to constituents that contribute to the modulation of the oxidative balance *in vivo*. Various plant originated gastroprotectors have been used in clinical and folk medicine due to their beneficial effects on the gastric mucosa. Literature has centered primarily on their pharmacological action in experimental animals using different models of gastric lesions induction (Borelli and Izzo 2000; Zayachkivska et al. 2005; Olaleye and Farombi 2006). It was shown previously that stress with cold exposure and restraint for 3.5 h induces severe gastric mucosal damage in rats.

In this study we used CRS experimental model to investigate the protective effect of OLE, a natural antioxidant, on gastric mucosal damage, since in this model the pathogenesis of the lesions has been related with production of ROS. Hence, the aim of the study was to evaluate the effect of an OLE on stress-induced histological changes, as well as biochemical perturbations, in this animal model.

Pretreatment with all three doses of OLE significantly ($p < 0.01$) reduced gastric lesions induced by CRS. CRS caused minimum of gastric lesions and the best inhibition

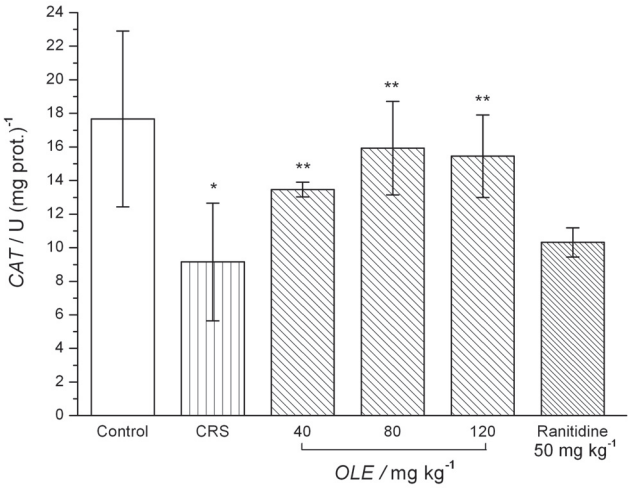

Figure 5. Effect of intragastric pretreatment with olive leaf extract (OLE) applied in graded doses ranging from 40 mg·kg^{-1} up to120 mg·kg^{-1} and ranitidine (50 mg·kg^{-1}) on the catalase (CAT) activity (U (mg prot)$^{-1}$) in gastric mucosa. * indicates statistical significance ($p < 0.05$) of difference in CAT activity in non-pretreated rats exposed to CRS as compared to the control animals. ** indicates statistical significance ($p < 0.05$) of difference in CAT activity in pretreated rats as compared to the CRS exposed rats without pretreatment.

of UI, related to the non-pretreated group, was obtained in animals pretreated with 80 mg·kg^{-1} of OLE (70%). Only gastric mucosal edema and petechiae were seen in almost

all (5 of 6) animals in this experimental group. The higher dose (120 mg·kg^{-1}) did not show the greatest effect, so the dose-response of three investigated doses of OLE was not obtained. We can only assume that dose-response could be obtained in experiments where the lowest dose investigated is 10 or 20 mg·kg^{-1}. Moreover, on the basis of our results we suppose that doses of 160 or 200 mg·kg^{-1} or higher, could cause pro-oxidative effects and consequently worse results, because it is happened in experimental studies of natural antioxidants (Lankin et al. 1999; Park and Lee 2008).

Macroscopic results were confirmed histologically. CRS exposed non-pretreated animals displayed a loss of superficial epithelium and necrosis of the upper mucosal layer, deep erosions and submucosal edema. In addition, a dense acute inflammatory cell infiltrate, consisting mainly of neutrophils, was noted. In striking contrast, gastric specimens from the OLE pretreated animals displayed minimal or no pathohistological changes. Only superficial erosions and vasodilatation were seen.

Since lipid peroxidation is a well-established mechanism of cellular injury (Kwiecien et al. 2002), we measured the changes in the MDA concentrations as an indicator of lipid peroxidation in gastric mucosa. Pretreatment with OLE (80 mg·kg^{-1} and 120 mg·kg^{-1}) significantly restricted rise in gastric MDA concentration caused by CRS. The results obtained in our study are in agreement with previously reported results. Thus, status of the antioxidant enzymes along with lipid peroxidation was studied in CRS-induced ulcers by Govindarajan et al. (2006), and increase in MDA concentration was noticed. A study conducted by Brzozowski et al. (2005) documented that MDA concentration is significantly increased in gastric mucosa exposed to ethanol or water immersion and restraint stress (WRS).

Antioxidant enzymes SOD and CAT, important cellular antioxidants, contribute to the gastric oxidative-antioxidative balance. Decrease of both SOD and CAT activity in gastric mucosa of immobilized rats leads to the accumulation of ROS and consequently to MDA concentration rise. In our investigation, CRS induced inhibition of SOD and CAT activity, suggesting important role of these enzymes in pathogenesis of stress ulcer disease. Other published results support this finding: SOD and CAT activity in rat stomach tissue was decreased by indomethacin- and HCl/ethanol-induced oxidative gastric mucosal damage (Olaleye and Farombi 2006), CAT activity decreased in CRS (Govindarajan et al. 2006); SOD activity is significantly decreased in 3.5 h of WRS (Brzozowski et al. 2005). In the present study, OLE administered to rats prior to stress induction attenuated inhibition of SOD and CAT activity, and thus implicated its role in modulation of the oxidative balance in gastric mucosal defense.

The antioxidative effect of total OLE most probably results from the ability of its constituents to scavenge ROS, produced in CRS, which initiate lipid peroxidation. Phytochemical analysis of OLE EFLA®943 performed by our laboratory shown high oleuropein content (almost 20%) and other constituents important for gastroprotection (apigenine-7-*O*-glycoside, luteolin-7-*O*-glycoside, quercetin, and caffeic acid), as well as, low concentration of tannins (Dekanski et al. 2009).

Previous studies shown that membrane lipid peroxidation was prevented by oleuropein, which exhibited strong antioxidant protection in oxidative stress during ischemia-reperfusion in experimental model of myocardial injury (Manna et al. 2004; Andreadou et al 2006).

Luteolin-7-*O*-glycoside is widespread in plant species, its anti-radical activity is well known, and its anti-ulcer activity was previously confirmed (Borelli and Izzo 2000).

Also, it has been reported that quercetin prevent gastric mucosal lesions induced by ethanol. Possible mechanisms include inhibition of lipid peroxidation (Alarcon de la Lastra et al. 1994), inhibition of the gastric proton pump (Di Carlo et al. 1999), and scavenging of free radicals associated with a significant enhancement in glutathione peroxidase activity (Martin et al. 1998).

Radical scavenging abilities for apigenine-7-*O*-glycoside and for caffeic acid were also reported (Benavente-Garcia et al. 2000).

Furthermore, recent study demonstrated gastroprotective effect of oleanolic acid (OA) derivatives (Sanches et al. 2006). A single oral administration of OA derivatives at the selected concentrations inhibited the appearance of gastric lesions induced by ethanol, aspirin and pylorus ligature. The effect of OA, which is important constituent of olive leaf and olive oil, was comparable with that of ranitidine at 50 mg·kg^{-1} and with that of omeprazole at 20 mg·kg^{-1} (Astudillo et al. 2002).

It is known that low concentration of tannins has role to „tan" the gastric mucosa and to render it less permeable and more resistant to chemical and mechanical injury or irritation (Asuzu and Onu 1990). Gastroprotective effect of tannins is experimentally confirmed when administration of tannins showed significantly lower stomach free radical concentrations in rats (Ramirez and Roa 2003). All of above mentioned effects of OLE constituents are of great importance in their possible synergistic effects in gastroprotection.

The results obtained indicate that the gastroprotective potential of OLE is probably related to its ability to maintain the cell membrane integrity, by its antilipid peroxidative activity that protects the gastric mucosa against oxidative damage, and by its ability to strengthen the mucosal barrier, the first line of defense against exogenous and endogenous ulcerogenic agents. The gastroprotective effect of OLE in fasted rats was similar to that obtained with antisecretory drug, ranitidine. Hence, we could not exclude the useful

role of antisecretory medications in the prevention of stress-related gastric mucosal damage, as acid and pepsin also contributed to the development of this condition. We infer that a combination regimen, including both antioxidants and antisecretory drugs, may be beneficial in preventing mucosal cell damage. In order to further elucidate the OLE mechanism of gastroprotective effect, our future investigation will be focused on other experimental models of mucosal injury and additional gastric mucosal defense mechanisms.

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