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INFLUENCE OF *VIBURNUM OPULUS* PROANTHOCYANIDINS ON STRESS-INDUCED GASTROINTESTINAL MUCOSAL DAMAGE

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Recent studies demonstrated that the proanthocyanidins (PA), the polymers of flavan-3-ols, naturally occurring plant metabolites widely available in fruits, vegetables, nuts, seeds, flowers and bark, have anti-inflammatory, anticarcinogenic, anti-allergic, antioxidant and vasodilatory actions. We hypothesized that *Viburnum opulus* PA (VOPA, *Caprifoliaceae*), due to activation of multifactorial gastrointestinal mucosal defense mechanisms, exert gastroduodenoprotective effects. The aim of the study was: 1) to investigate VOPA effects on gastroduodenal mucosal integrity and pattern of carbohydrate binding proteins and nitric oxide (NO) content in intact mucosa and that exposed to non-topical ulcerogens (stress) in rats without and with capsaicin (125 mg/kg, sc) denervation; and 2), to assess the role of activity of antioxidizing enzymes superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GPx) in VOPA-iduced gastroduodenoprotection against water immersion and restraint stress (WRS) in rats. VOPA was administered orally in dose of 25, 50 or 75 mg/kg body weight. Gastroduodenal mucosal damage detected by routine light microscopic investigation and lectin histochemistry set, purified from plant and animal sources of Carpatian region. NO content, pro-and antioxidant system were determinated by routine laboratory methods. Pretreatment with VOPA afforded gastroduodenoprotection and was accompanied by an increase in NO expression, both changes being reversed by sensory denervation, as well as by the rise of SOD, CAT activity and fall in MDA content.
Our study shows that VOPA exerts a potent gastroduodenoprotective activity *via* an increase in endogenous NO generation, suppression of lipid peroxidation and mobilization of antioxidant activity and changes in glycoconjugate content of the gastroduodenal mucosa of rat.

Key words: *gastroduodenal mucosa, proanthocyanidin, stress, antioxidant defence, lipid peroxidation, lectin histochemistry*

INTRODUCTION

Acid-related disorders are currently considered as important and widespread diseases (1). The data from modern studies indicate that universal type of injury includes an oxidative stress which triggers membrane leakage, release of reactive oxygen (ROS) and nitrogen reactive species followed by induction of peroxidative reactions that result in molecular damage and release of metals with extension of free radicals discharge (2 – 4). The ulcer-promoting inductors involve changes in intercellular signal pathways and are mediated by the influence of endothelial-depended action of the nitric oxide (NO), prostaglandin (PG)-cyclooxygenase (COX) and antioxidant systems and other components of cell defense system (5 – 7). ROS play a key part in the multiple step process leading to formation of gastric and esophageal adenocarcinoma (8, 9). Significant role in cellular homeostasis is played by glycoconjugates (glycoproteins, proteoglycans, glycolipids) which can modulate the function of structures to that they are conjugated and, therefore, can affect cell integrity, including epithelial cell spreading, migration, proliferation and differentiation. Currently structure-function relationships of carbohydrates attached to cell surface proteins and lipids are presented as an effective high-density information system and pattern of produced a glycan profile (glycome) is as characteristic as a fingerprint (10). Moreover, a rapid modification during dynamic processes presented in oligosaccharide determinants is carried out by lectins (11). Simultaneously, recent investigations have reported interest that the bioflavonoids act as natural cytoprotectors and hemopreventive agents (12–14). Bioflavonoids are widely distributed in higher plants and are an integral part of the human diet. The potential use of bioflavonoids in preventing, reducing and/or delaying various lesions is due to their antioxidant and antiradical activity (15–18). Important but until now overlooked group of bioflavonoids are proanthocyanidins (PA) in nature are present in fruits, berries, vegetables, nuts, seeds, flowers, and bark in many plants (19, 20). The therapeutic effects of PA were shown to involve radical scavenging, quenching, and enzyme-inhibiting actions (21–23). There is also evidence of antibacterial, antiviral, anticarcinogenic, anti-inflammatory, anti-allergic, and vasodilatory actions of these compounds (24–27). In addition, they were found to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and to affect enzyme systems including phospholipase A2, cyclooxygenase, and lipoxygenase (28–30). Based on these reported findings, PA may be useful component in the treatment of a number of conditions. At present PA are refined mostly from grape seeds (*Vitis vinifera*) and the white pine (*Pinus maritima, P. pinaster*) in southern Europe, whereas PA in eastern Europe are obtained from apples, berries, barley, rhubarb, rose hips and guelder rose (31). Recent studies showed that the most frequently occurring PA are contained in different species in the genera *Viburnum* from family *Caprifoliaceae* (32 – 34).

In Ukraine and other countries from East Europe *Viburnum opulus L.*, also known as the guelder rose or the snowball tree, popular cultivated species, is

commonly used in traditional folk medicine. We hypothesized that *Viburnum opulus* PA (VOPA) exert gastroduodenoprotective effects due to the activation of multifactorial gastrointestinal mucosal defense lines.

The aim of the present study was: 1) to investigate the effect of pretreatment with VOPA on acute gastroduodenal lesions induced by non-topical ulcerogens (water immersion and restraint stress, WRS) and to detect the role of NO, sensory nerves and changes in pattern of the reactive glycoconjugates in gastroduodenal mucosa of intact and exposed to induced damage rats; 2) to determine the mechanism of VOPA gastroprotection related to activity of antioxidizing enzymes superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GPx) and pattern of carbohydrate binding proteins.

MATERIAL AND METHODS

Male Wistar rats, weighing 180-220 g and fasted for 24 h with free access to water were used in our studies. These experimental procedures were approved by the University Ethical Committee for Animal Research. All trials followed University Guide for Care and Use of Laboratory Animals that run in accordance to the statements of European Union regarding handling of experimental animals.

Gastroprotection studies

Acute gastric lesions were induced by the WRS when the animals were placed in restraint cages and immersed vertically to the level of the xiphoid process in a water bath of 23° for 3.5 h. For determination of VOPA influence gastroprotection and sensory afferent nerves in mechanism of WRS-induced gastric lesions were induced in rats with capsaicin induced deactivation of these nerves 2 weeks before experiment. Capsaicin (Sigma Co., USA) was used at doses of 25 and 50 mg/kg injected subcutaneously for 3 consecutive days. All injections of capsaicin were performed under ether anesthesia to counteract the pain reactions and respiratory impairment associated with injection of this agent. In studies on gastroprotection induced by VOPA (plant extraction procedure were performed from air-dried berries of *Viburnum opulus L* as described elsewhere) given intragastrically, following groups of rats were used: 1) control (Cont) – vehicle (1 ml saline *per os*) 2) VOPA 1 - 25 mg/kg body weigh (BW), 3) VOPA2 – 50 mg/ kg BW, 4) VOPA3 – 75 mg/ kg BW, 5) VOPA 4 – 50 mg/kg BW (on rats with sensory denervation), 30 min prior to introduction WRS.

After the end of experiment rats were anaesthetized and venous blood was collected. Then the animals were sacrificed and the stomach was immediately removed for macroscopic analysis, opened along the greater curvature and placed flat to count the number of gastric lesions by two investigators, unaware of the treatment given. The stress lesions were defined as round or linear mucosal defects of at least 0.1 mm in diameter.

Lectin labeling

For microscopic analysis segments of the glandular region of stomach and proximal region of duodenum were excised and used for the routine histological examination and the lectin histochemistry. The lectin set included peanut agglutinin (PNA, specific to βDGal→3DGalNAcDGal), *Helix pomatia* agglutinin (HPA, specific to DGalαNAc), wheat germ agglutinin (WGA, specific DGlcNeuNAc) conjugated to peroxidase (purchased from “Lectinotest

Lab", Ukraine). Lectin label was visualized with diaminobenzidine (DAB) in PBS as described elsewhere (10, 11, 35). All incubation procedures were conducted at room temperature. Images of histological slices were investigated using a digital video camera connected to a microscope (MBI-15-2, LOMO, Russia) and were processed using the AVerMedia FZC Capture image analysis program (AVerMedia Technologies, Inc., USA) and carried out by semi-quantitative optical analysis, taking account the intensity, being considered as absent (–), weak (+), moderate (++) or intense (+++).

Determination of total nitrate and nitrite concentration

Nitrate/nitrite (NO_2^- and NO_3^-) amounts (NOx) in plasma and erythrocytes hemolysate were determined using Griess reagent (36). Sample proteins were sedimented by 30% ZnSO4. After the centrifuging the supernatant was incubated with metal cadmium for 12 hours thereby reducing nitrate to nitrite. Then Griess reagent was added, and total NOx was measured at 550 nm spectrophotometrically (SOLAR, Model PV 125 1C) and expressed in terms of microM.

Determination of lipid peroxidation (LPO)

Malondialdehyde (MDA) was assessed by the method of Timirbulatov R., *et al.* (37). Briefly, 0,1M standard phosphate solution (SPS) in pH 7,4 was added to 0, 1 mM $KMnO_4$ and 10 mM $FeSO_4$ to the homogenate and incubated for 10 minutes at room temperature (RT), followed by boiling with 20% acetic acid and 0,6% thiobarbituric acid for 60 minutes in a water bath. On cooling, butanol pyridine was added and centrifuged for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of microM/mg.

Determination of SOD activity

SOD activity was measured by the method of Kostuk *et al.* (38) that was based on determination of the degree of inhibition oxidation reaction of quercetin. Autooxidation of quercetin was performed in 0.015 M SPS, containing 0.1 mM EDTA; 0,8 M tetramethylendiamid (TMDA) in final volume of 3,5 ml. Reaction was induced by administration to quercetin inbubation environment 0,1 ml dimethylformamid (DMFA). Activity was expressed as the amount of enzyme that inhibited the reaction by 50%, which is equivalent to one unit and results are expressed as units (U).

Determination of CAT activity

The activity of CAT was determined using the method described by Koroluk (39). Briefly, principle of method consists in ability of peroxide oxide formed with molybdenum salts stable colored complex. This reaction yields a stable chromophore with maximal absorbance at 410 nm in a spectrophotometer. Results were expressed in terms of microM of degraded H_2O_2/mg×h.

Determination of glutathione peroxidase (GPx) level

GPx activity was determined as described by Moin (40). Briefly, activity of GPx depended from oxidation rate of glutation in the presence of protein precipitation. Formation color reaction was result of response of SH-group with 5,5-ditiobis(2-nitrobensoic) acid (DTNNA) with development colored product – tionitrophenolic anion. The level of this product was directly proportional to level of SH-groups that react with DTNNA. The level of reduced form of glutathione (GSH) before and after incubation was measured by spectrophotometer. Results were expressed as micromole GSH/mg×h.

Statistical analysis

Statistical analysis was performed with program package STATISTICA for Windows 5.5 (Stat Soft, USA). The results of evaluations according the semiquantitative scale are expressed as means ± SEM. For comparison of data used paired Newman-Keuls's test with a level of significance at $P < 0,05$.

RESULTS

In the 1st series of experiments the effects of intragastric (ig) pretreatment with vehicle or with VOPA applied in graded doses ranging from 25 to 75 mg/kg on the mean area of gastroduodenal lesions induced by WRS and the accompanying changes in the NOx were detected (*Fig. 1*). Pretreatment with VOPA (50 mg/kg ig) in rats with intact vagal afferents significantly reduced gastroduodenal lesions induced by WRS and activated NO system but this dose applied to the rats with capsaicin denervation, failed to exhibits gastroprotective effect and the amount of NOx was significantly decreased.

In the 2nd series of experiments the state of pro- and antioxidant balance in the gastric mucosa of rats exposed to WRS without or with pretreatment with graded doses of VOPA was determinated. The results are shown in *Table 1*. The mucosal

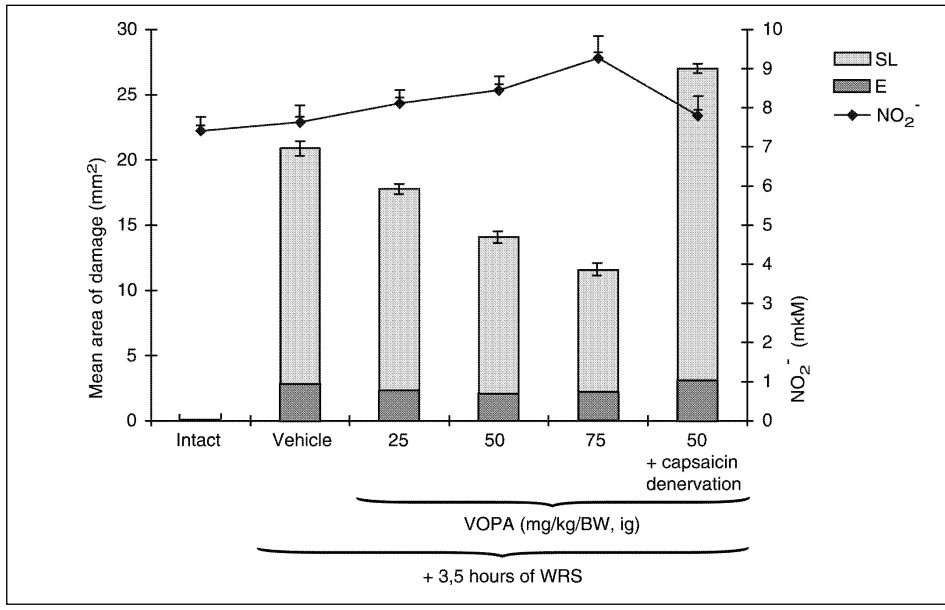

Fig. 1. Mean area of WRS-induced gastroduodenal lesions and NO_x content in rats without (vehicle only) and with pretreatment with graded doses of VOPA without and with sensory denervation by neurotoxic doses of capsaicin (125 mg, sc). SL – superficial lesions, E - erosions

level of concentration of MDA in intact animals averaged 56,75±2,50 microM/mg. The MDA concentration, as an index of PLO, was significantly increased in vehicle-treated rats with gastric mucosa exposed to WRS as compared to that in the intact gastric mucosa. Pretreatment with VOPA applied ig in a dose of 25 mg/kg/BW or higher, significantly attenuated the MDA concentration in animals exposed to WRS (*Table 2*). The mucosal SOD activity was inhibited in the gastric mucosa of animals exposed to WRS as compared to the respective value in intact gastric mucosa. In comparison, the pretreatment with VOPA applied ig in graded doses (25, 50 and 75 mg/kg/) significantly increased SOD activity and dose-dependently reversed the harmful effect of WRS, showing opposite effect on the MDA content. The similar dynamics of effects with activity of CAT and reduced form of glutathione (GSH) in gastric mucosa as well as in that pretreated with vehicle (saline) and graded doses of VOPA in the gastric mucosa of rats exposed to WRS were detected. A significant decrease of CAT and GSH values were found in rats exposed to 3.5 h of WRS alone (control group), when compared with that in intact mucosa. In VOPA1 group administration of VOPA (25 mg/kg ig), before the WRS, resulted in a significant increase of CAT and GSH when compared with that recorded in rats exposed to 3.5 h of WRS. Pretreatment with VOPA in 50 mg/kg ig significantly

Table 1. Concentration of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GPx) in the gastric mucosa of rats exposed to 3.5 h of water immersion restraint stress (WRS) without and with applied proanthocyanidins of *Viburnum opulus* (VOPA) in graded doses ($VOPA_1$ - 25 mg/kg/bw, $VOPA_2$ – 50 mg/kg/bw, $VOPA_3$ -75 mg/kg/bw). Results are mean ±SEM of 8-10 rats. Asterisk (*) indicates a significant change as compared with the value obtained in intact gastric mucosa (intact group). Dagger (†) indicates a significant change as compared with the value obtained in rats exposed to 3.5 h of WRS. Double dagger (‡) indicates a significant change as compared to the value obtained in rats pretreated with VOPA in dose 50 mg/kg. Section sign (§) indicates a significant change as compared to the value obtained in rats pretreated with VOPA in dose 50 mg/kg/bw.

Index	Experimental group				
	Intact	Vehicle	$VOPA_1$	$VOPA_2$	$VOPA_3$
MDA, microM/mg	56,75±2,50	141,31±3,60*	120,93±0,69*†	111,43±0,81*†‡	108,68±0,58*†‡
SOD, U	269,85±0,68	255,31±2,17*	284,69±0,65*†	296,48±0,38*†‡	303,23±0,35*†‡§
CAT, microM H2O2 /mg×h	46,21±0,86	30,71±0,77*	32,76±0,18*†	37,13±0,23*†‡	41,76±0,33*†‡§
GPx, microM GSH/mg×h	2,92±0,01	1,81±0,02*	2,05±0,02*†	2,26±0,01*†‡	2,55±0,02*†§

* $p<0,05$ compared to intact
† $p<0,05$ compared to vehicle
‡ $p<0,05$ compared to $VOPA_1$
§ $p<0,05$ compared to $VOPA_2$

Table 2. Effect of proanthocyanidins of *Viburnum opulus* (VOPA) pretreatment on intensity of different specify of lectin-carbohydrate interaction in intact, WRS – induced damage gastric mucosa without and with VOPA pretreatment (50 mg/kg/body weigh) due to semi-quantitative analysis (absent "-", weak "+", moderate "++" or intensive reaction "+++").

Experimental groups	Intact		WRS		WRS + VOPA	
Gastric mucosa / Lectins and their specify of carbohydrates	Epithelial cells	Gland cells	Epithelial cells	Gland cells	Epithelial cells	Gland cells
Peanut agglutinin, PNA, βDGal specif	+++	+++	–	+	+	–
Helix pomatia agglutinin, HPA, DGalαNAc specif	++	++	+++	+++	++	+
Wheat germ agg;utinin WGA, DGlcNAc specif	+	+++	+++	++	++	+

enhanced the activity of CAT and GSH, when compared to that was recorded in vehicle-pretreated rats, but adding the VOPA in dose 75 mg/kg/bw ig markedly increased the mucosal CAT and GSH level, when compared to the value observed in VOPA2 and VOPA3 groups (*Table 1*).

Macroscopic and microscopic examination did not show any damage of the gastroduodenal mucosa in intact rats. Some abnormalities in epithelial morphology and microcirculation were seen in hematoxylin and eosin-stained sections from glandular region of stomach and proximal part of duodenum in vehicle-treated rats. Epithelial layer exhibited superficial and erosive lesions with signs of dystrophia, necrosis and apoptosis. Local hemodynamic disorders induced signs of irregular hyperemia, stasis and restricted perivascular diapedesis with hemorrhage. Intact rat epithelium of stomach was composed of mucosal cells with cuboidal shape and tight junctions between cells and with underlayered structures, without visible defects. As a result of stress reaction, epithelial cells undergo partial or full changes such as separation from lamina propria and destruction of intercellular contacts comparing with intact animals. As shown in *Fig.1*, the pretreatment with VOPA applied orally in the graded doses (25, 50, 75 mg/kg) caused a considerable decrease of WRS-induced gastroduodenal damage as compared to vehicle-treated rats. Administration of VOPA in a dose of 50 mg/kg also markedly reduced gastroduodenal mucosal damage induced by WRS.

Lectin histochemistry of stomach and duodenal mucosa of intact and WRS-induced rat without and with VOPA pretreatment revealed a marked heterogeneity of PNA, HPA and WGA binding. The corpus-fundus regions of

stomach showed differential lectin-binding patterns in all parts of the gastric gland area and epithelial barrier in intact and WRS-exposed animals (*Fig. 2*). Analysis of PNA, HPA and WGA label is presented in *Table 2*. PNA and WGA reactivity was restricted to surface epithelium, while these same lectins labeled mucous neck and gland cells in VOPA treated rat when compared to that recorded in vehicle-pretreated rats. These effects appeared simultaneously with the reduction in edema and alterations in gastric mucosa. Weak reaction with PNA, HPA, WGA lectin binding pattern for epithelial cells and complete loss of PNA staining for glandular cells and thus in the carbohydrate composition have been noted between gastric mucosa of VOPA treated WRS rat in comparison to the control. Intensive expression of HPA binding on apical surface of epithelium during WRS were determined by observation of essential changes in mucus gel layer glycoprotein content.

Changes in carbohydrate moieties of glycoconjugates revealed a strong positive reaction to HPA and PNA lectins in the goblet cells of duodenum presented in the WRS VOPA-treated animals when compared to intact control group (*Fig. 3*).

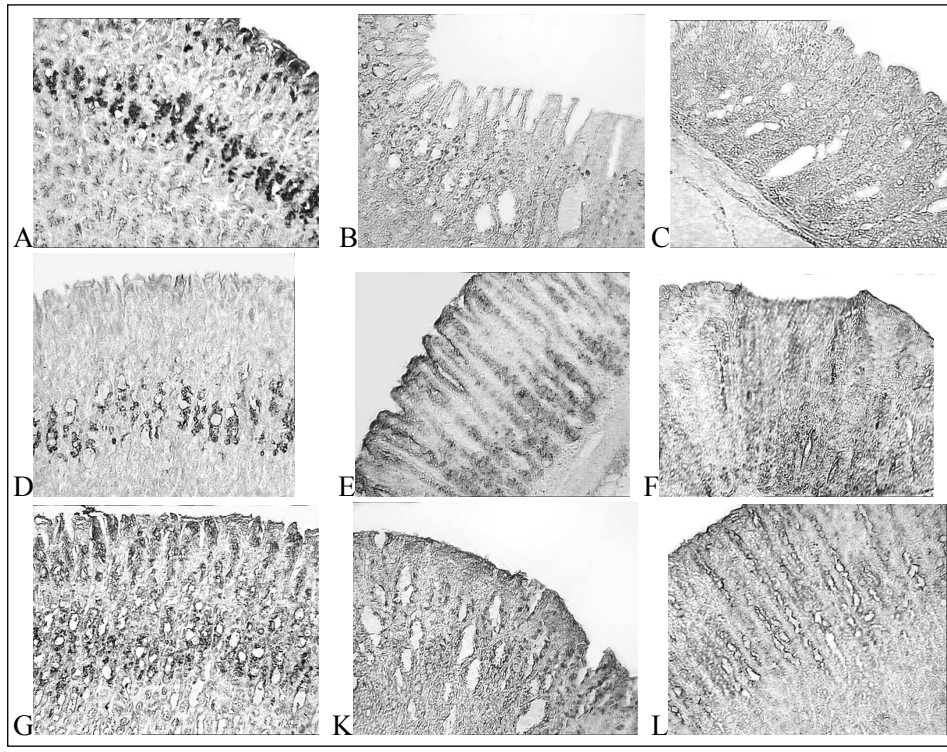

Fig. 2. Dynamic changes in pattern of expression of PNA (A, B, C), HPA (D, E, F) and WGA lectin receptors in gastric mucosa in intact (A, D, G) and vehicle (B, E, K) and VOPA-treated (C, F, L) rats (magnification x 300).

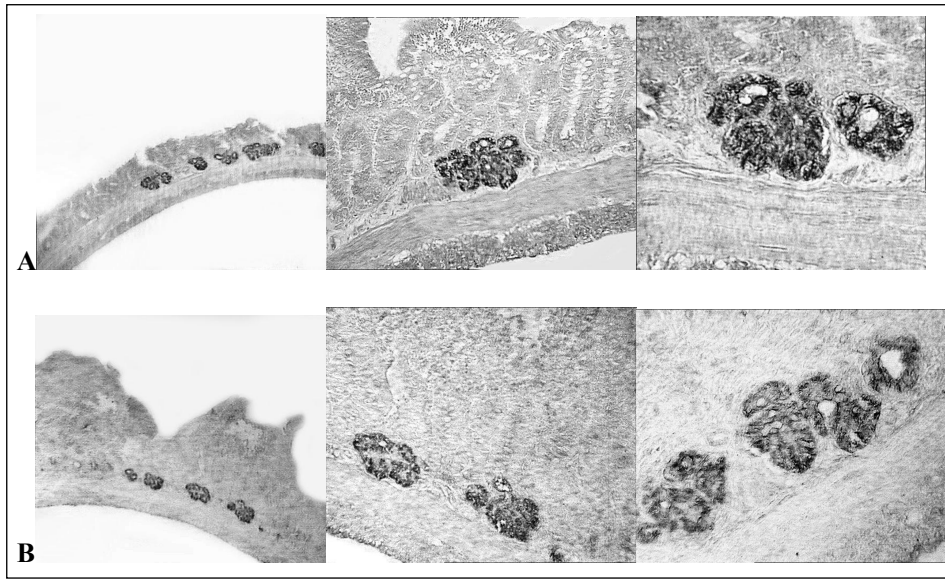

Fig. 3. Dynamic changes in pattern of expression of HPA (A, magnification x 120; 300; 600), PNA (B, magnification x 120; 300; 600) in duodenal mucosa in VOPA-treated (50 mg/kg/ body weight) rats.

DISCUSSION

The present studies were performed to examine the influence of PA on gastroduodenoprotection. It is generally accepted that oxidative stress is accompanied by production of free radicals, enhanced lipid peroxidation and an impairment of antioxidizing enzyme activity. Furthermore, the increased generation of cytokines, such as IL-1β and TNFα, appears to contribute to the pathomechanisms of stress-induced lesions of gastrointestinal mucosa (41). The free radical scavenging abilities of PA were well recognized (42, 43). The attenuation of acid-dependent lesions such as caused by WRS can involve endogenous gastric mucosal generation of prostaglandins (PGE2), derived from COX activity, NO mediated vasodilatation, suppression of lipid peroxidation and antiradical activity (44). Many observations have demonstrated that PA positively affect the vasoprotection. *In vivo* studies have shown that PA from grape seed extract are stronger free radical scavengers and inhibitors of oxidative tissue damage than vitamin C, vitamin E succinate, vitamin C and vitamin E succinate combined, and beta carotene (18, 30, 45). Moreover, *in vitro* experimental results have verified that PA have specificity for the hydroxyl radicals in addition of having the ability to non-competitively restrain the activity of xanthine oxidase, a most important producer of free radicals, elastase, collagenase, hyaluronidase, and beta-glucuronidase (46). Importance of NO in

the mechanism of gastric integrity is supported by our earlier observations that extract prepared from Amaranth seed rich of bioflavonids exhibit gastroprotection against the gastric injury induced by absolute ethanol predominantly due to the input PG/COX, NO and sensory nerves (47). To clarify the possible mechanism of VOPA by which gastroduodenal lesions were attenuated, we examined changes in NOx and lipid peroxide levels. After the demonstration that the application of VOPA dose-dependently increases NOx production and attenuates the gastric and duodenal lesions induced by WRS *via* increased gastric microcirculation and mucus secretion and reversed in capsaicin-treated rats, we conclude that PA are potent and important gastroduodenoprotective natural substances. These observations also confirm by results in the recent study of extract of one related species, i.e. *Viburnum awabuki,* modulating the NO synthesis or expression has been considered potential anti-inflammatory and cancer chemopreventive agents (46). As shown by investigation of the fruit of *Viburnum dilatatum Thunb*, called gamazumi, having radical scavenging properties, enhanced antioxidant activity may be the key mechanism contributing to physiological effects of PA (48). Our results emphasized that the protective activity of VOPA against ulcerogenesis was realized through the suppression of ROS and decreased expression by tissue concentration of MDA. PA have also demonstrated preferential binding to areas characterized by a high content of glycosaminoglycans (epidermis, capillary wall, gastrointestinal mucosa, etc.) (11). This feature makes them useful in decreasing vascular permeability and enhancing capillary integrity, vascular function and peripheral circulation and increasing resistance of epithelial barrier of gastroduodenal mucosa (49). Our study showed for the first time that the exposure of rat gastroduodenal mucosa to WRS without and with VOPA application, leads to modification in their glycoconjugates composition in epithelial cells and suggests that PNA, HPA and WGA lectins are promising histological markers to differentiate glycoconjugates as the integral components of cell membrane that regulate cell-to-cell interactions and take part in gastroduodenoprotection. We observed that pretreatment with VOPA resulted in modification of glycoconjugates in cell-surface membranes and intercellular contacts that prevented destructive action on mucosal cells and abolished the gastric and duodenal lesions induced by WRS. Reliable variation of amount of PNA and HPA labeling comparison with the intact mucosa was observed in WRS-induced injury suggesting the depolymerization of glycoprotein layer of epithelial barrier (50, 51). Application of VOPA promoted restoration of the gastroduodenal glycoprotein content that testifies about increase of mucus gel layer resistance and gastric mucosal resistance. In summary, we conclude that VOPA application exhibits preventive effects gastroduodenal damage induced by WRS and this requires future study to provide evidence that VOPA, indeed, increases the gene and protein expression of NOS and promotes the changes relevant not only in modification of cell-surface and intercellular

glycoconjugates but also of intracellular glycoconjugates. This could form an interesting area for future investigation towards potential use of PA in treatment acid-related diseases in humans.

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