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ROLE OF SENSORY AFFERENT NERVES, LIPID PEROXIDATION AND ANTIOXIDATIVE ENZYMES IN THE CARBON MONOXIDE-INDUCED GASTROPROTECTION AGAINST STRESS ULCEROGENSIS

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Carbon monoxide (CO) is a physiological gaseous mediator recently implicated in the mechanism of gastric mucosal defense due to its vasodilatory and antioxidative properties. Small quantities of endogenous CO are produced during heme degradation by heme oxygenase (HO-1), however, the involvement of the capsaicin-sensitive afferent neurons releasing calcitonin gene related peptide (CGRP) and anti-oxidative factors and mechanisms in the CO-induced gastroprotection against stress ulcerogenesis has been little studied. We investigated the possible role of CO released from the CO donor, tricarbonyldichlororuthenium (II) dimer (CORM-2) in the protection against water immersion and restraint stress (WRS)-induced lesions in rats with intact sensory nerves and those with capsaicin denervation and the accompanying changes in malondialdehyde (MDA) content considered as an index of lipid peroxidation, the activity of GSH and SOD-2 and gastric mucosal expression of antioxidative enzymes glutathione peroxidase (GPx) and SOD-2. Wistar rats with intact sensory nerves or those with capsaicin administered in total dose of 125 mg/kg s.c. within 3 days (capsaicin denervation) were pretreated either with 1) vehicle (saline) or 2) CORM-2 (1 mg/kg i.g.) with or without exogenous CGRP (10 μg/kg i.p.) and 30 min later exposed to 3.5 h of WRS. At the termination of WRS, the number of gastric lesions was counted and gastric blood flow (GBF) was assessed by H_2-gas clearance technique. The mucosal content of MDA and reduced glutathione (GSH) and the activity of SOD-2 were determined and the expression of GPx-1 and SOD-2 mRNA in the gastric mucosa was analyzed by real-time PCR. The exposure of rats to 3.5 h of WRS resulted in numerous hemorrhagic gastric lesions and significantly decreased the GBF, raised MDA content and significantly decreased the mucosal SOD and GSH contents compared with intact gastric mucosa and these changes were exacerbated in rats with capsaicin denervation. Pretreatment with CORM-2 (1 mg/kg i.g.) which in our previous studies significantly reduced the ethanol and aspirin-induced gastric damage, significantly decreased the number of WRS-induced gastric lesions while raising the GBF and significantly increasing the activity of SOD and GSH ($P < 0.05$). The pretreatment with CORM-2 significantly decreased MDA content as compared with vehicle-pretreated rats exposed to WRS ($P < 0.05$). The reduction of WRS damage and the accompanying increase in the GBF as well as the significant decrease in MDA content and the increase in GSH content and SOD activity induced by CORM-2 (1 mg/kg i.g.) were all significantly altered in rats with capsaicin denervation ($P < 0.05$). The concurrent treatment of CORM-2 with exogenous CGRP in rats with or without sensory nerves tended to decrease the number of WRS lesions as compared with CORM-2 alone pretreated animals and significantly increased the GBF over the values measured in gastric mucosa of CORM-2 alone pretreated rats with or without capsaicin denervation. Such combined administration of CORM-2 and CGRP in rats with capsaicin denervation significantly inhibited an increase in MDA and 4-HNE content and evoked a significant increase in the GSH concentration ($P < 0.05$) remaining without significant effect on the increase in SOD activity observed with CORM-2 alone. The gastric mucosal expression of SOD-2- and GPx-1 mRNA was significantly increased as compared with those in intact gastric mucosa ($P < 0.05$). The pretreatment with CORM-2 applied with or without CGRP failed to significantly alter the mRNA expression for SOD-2 and GPx in the gastric mucosa of rats exposed to WRS. Both, the expression of SOD-2- and GPx-1 mRNA was significantly increased in capsaicin denervated rats exposed to WRS rats ($P < 0.05$) and this effect was abolished by the pretreatment with CORM-2. The expression of SOD-2 tended to decrease, though insignificantly, in rats pretreated with the combination of CORM-2 and CGRP as compared with that detected in CORM-2 alone in rats with capsaicin denervation. In contrast, the mRNA expression of GPx-1 was significantly decreased in gastric mucosa of capsaicin-denervated rats treated with the combination of CORM-2 and CGRP as compared with CORM-2 alone pretreated animals. We conclude that 1) CORM-2 releasing CO exerts gastroprotective activity against stress ulcerogenesis and this effect depends upon an increase in the gastric microcirculation and the vasodilatory activity of this gaseous mediator, and 2) the sensory nerve endings releasing CGRP can contribute, at least in part, to the CO-induced gastric hyperemia, the attenuation of gastric mucosal lipid peroxidation and prevention of oxidative stress as indicated by the CORM-2-induced normalization of the antioxidative enzyme expression enhanced in gastric mucosa of capsaicin-denervated rats.

Key words: *carbon monoxide, stress damage, sensory nerves, gastric blood flow, lipid peroxidation, malondialdehyde, capsaicin, superoxide dismutase, glutathione*

INTRODUCTION

Carbon monoxide (CO) is a gaseous vasodilatatory mediator with recognized anti-inflammatory, cardioprotective and antiapoptotic properties (1). Under physiological conditions CO is formed *via* metabolic pathway of heme degradation by the enzymatic activity of heme oxygenase (HO)-1 (2). CO exerts the gastric vasodilatation mediated by the activation of potassium channels, the second cellular messenger, guanylyl cyclase/cGMP and MAP-kinase system (3). Previous studies revealed that CO may exert the vasoactive, antithrombotic and antiproliferative effects (4, 5). The tricarbonyldichloro-ruthenium (II) dimer $[Ru(CO_3)Cl_2]_2$ (CORM-2, *Fig. 1*) elicits biological effects due to its CO-releasing ability (1, 6, 7). Besides nitric oxide (NO) and hydrogen sulfide (H_2S), also CO contributes in the mechanism of gastric mucosal defense against gastric damage induced by ethanol, alendronate and stress (2, 8, 9). However, the involvement of CO in the pathogenesis of acute water immersion and restraint stress (WRS)-induced gastric lesions with focus to an involvement of sensory afferent nerves releasing calcitonin gene related peptide (CGRP) (10-12), lipid peroxidation and the antioxidative factors indices of the gastric mucosa oxidative stress, such as the activity and the gastric mucosal expression of superoxide dismutase (SOD) and glutathione peroxidase (GPx), have been little studied.

Previous studies demonstrated that the integrity of the gastric mucosa depends on a variety of protective factors including the gastric microcirculation regulated by the activity of capsaicin-sensitive sensory fibers (11, 12) *via* release from their endings of vasodilatory mediators such as calcitonin gene related peptide (CGRP) and substance P (10). Capsaicin is known to serve as the tool for determination of sensory nerve activity, because when applied in a low dose, this agent has been reported to cause sensory nerves activation accompanied by the release of vasoactive neurotransmitter CGRP, whereas the application of a high doses of capsaicin induces the functional ablation of sensory nerves resulting in a depletion of CGRP from these nerve endings (12, 13). We and others have reported that WRS led to increased oxidative metabolism as manifested by augmentation of lipid peroxidation and the neutrophils infiltration of gastric mucosa (14). Neutrophils have been considered as the source of superoxide radical anion ($O_2^{\bullet -}$) having the most toxic influence on gastric mucosa within the reactive oxygen species (ROS). The $O_2^{\bullet -}$ reacts with cellular lipids, leading to the formation of lipid peroxides further metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (15). It has been demonstrated that superoxide dismutase (SOD) acts as a major antioxidative enzyme, known to scavenge ROS and prevent their cell destructive action (16). However, the role of sensory nerves releasing CGRP, the lipid peroxidation and SOD activity in the mechanism of CO-induced protection against WRS-induced gastric damage is largely unknown and has not been studied in detail.

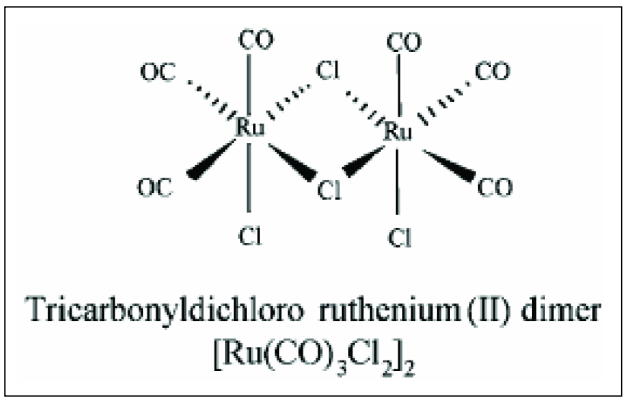

Fig. 1. Structure of CORM-2 (tricarbonyldichloro ruthenium (II) dimer $[Ru(CO_3)Cl_2]_2$) [adapted from Motterlini R *et al.* (1)].

Therefore, the aim of our present study was 1) to determine the role of capsaicin-sensitive nerves and gastric blood flow in CORM-2-induced gastroprotection against WRS ulcerogenesis and 2) to assess whether depletion of CGRP in rats with capsaicin denervation as well as the replacement of CGRP deficit by replacing it with exogenous CGRP administration in these animals would have alter the potential protective and blood flow effects of CORM-2, and 3) to evaluate whether the pretreatment with CORM-2 affects the accompanying changes in lipid peroxidation determined by means of malondialdehyde (MDA) concentration, the SOD-2 and reduced glutathione (GSH) contents and the mucosal expression of SOD-2 and GPx-1 mRNAs in rats with or without the capsaicin-induced functional ablation of sensory nerves exposed to onset of stress.

MATERIAL AND METHODS

Ninety-five male Wistar rats, weighing about 200 g and fasted for 24 h before each experiment, were used in all studies.

The experimental design was approved by the Ethical Committee at Jagiellonian University Medical College in Cracow and all experiments were run in accordance with statements of the Helsinki Declaration regarding handling of experimental animals.

Experimental design, production of gastric lesions and capsaicin denervation

The animals were divided into 6 major experimental groups A – F. Groups A – C underwent the procedure of deactivation of sensory nerves by using capsaicin (capsaicin denervation) at about 2 weeks before the start of WRS experiments. For this purpose, the animals of groups A - C were treated with capsaicin (Sigma Co., St Louis, USA) injected subcutaneously (s.c.) for 3 consecutive days at a dose of 25, 50 and 50 mg/kg (total dose of 125 mg/kg). All injections of capsaicin were performed under isofluran anesthesia to counteract the pain reaction and respiratory impairment associated with injection of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective wiping movement was counted as described previously (17).

In group A, rats with the ablation of sensory nerves received vehicle (DMSO/saline solution) and 30 minutes later they were exposed to 3.5 h of WRS at 23°C, according to the method originally proposed by Takagi *et al.* (18) and modified and described by our group elsewhere (17). In groups B and C, rats with capsaicin-denervation were pretreated either with CORM-2, 1 mg/kg intragastrically (i.g.) alone, which in our recent study (8) significantly reduced the formation of stress-induced gastric damage or the combination of exogenous CGRP applied intraperitoneally (i.p.) in a dose of 10 µg/kg and CORM-2 (1 mg/kg i.g.) and after 30 min these rats were exposed to 3.5 h of WRS. In experimental groups D and E, rats with preserved function of sensory nerves (without capsaicin denervation) were pretreated with vehicle (DMSO/saline solution) or CORM-2 (1 mg/kg i.g.) 30 min before exposing them to 3.5 h of WRS. Group F used for our biochemical analysis and molecular determinations served as an internal control group and did not undergo any treatment.

Determination of gastric blood flow and number of lesions

The evaluation of gastric lesions and gastric blood flow (GBF) was performed at termination of 3.5 hours of WRS. To measure GBF the laser Doppler flowmeter (Laserflo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, Minnesota, USA) was employed. The animals were anaesthetized with pentobarbital 50 mg/kg (Biowet, Pulawy, Poland), then the abdomen was opened and the stomach was exposed to determine the GBF. The GBF was measured on the anterior and posterior walls of the stomach not involving gastric lesions. The mean values of three measurements were calculated and expressed as percent change from value recorded in intact mucosa. The number of gastric lesions were determined by computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) as described elsewhere (18).

Determination of lipid peroxidation

In order to determine the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), about 200 mg of gastric mucosa was excised from rats with or without capsaicin-deactivated sensory nerves pretreated with vehicle, CORM-2, the combination of CORM-2 and CGRP and 30 min later exposed to WRS. The gastric mucosa was mixed with 20 µl of 0.5 M BHT (butylated hydroxytoluene) in order to prevent sample oxidation. Samples were subsequently homogenized in 20 mM Tris (pH = 7.4) for 15 s. Next, the homogenates were centrifuged for 10 min (3000 g at 4°C). The obtained clear supernatant was stored at 80°C prior to testing.

The colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxis, Portland, USA) was used to determine of MDA and 4-HNE tissue concentrations. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and 4-HNE at 45°C. As the result, this reaction yields a stable chromophore with maximal absorbance at 586 nm, which can be measured by spectrophotometry (Marcel s300, Warsaw, Poland). Results were expressed as nanomoles per gram of tissue (nmol/g) (19).

Measurement of gastric mucosal glutathione content

For a measurement of the concentration of reduced form of glutathione (GSH), the gastric mucosal sample of 200 mg was collected and processed in manner similar to that described above for determination of lipid peroxidation. Then 5% aqueous solution of metaphosphoric acid was added to the sample in order to evoke protein precipitation. Then colorimetric assay for assessment of reduced glutathione concentration (Bioxytech, GSH-400, Oxis, Portland, USA) was used. The level of reduced glutathione was measured with maximal absorbance at 400 nm by spectrophotometer (Marcel s300, Warsaw, Poland). Results were expressed as micromole per gram of tissue (µmol/g) as described before (14, 16).

Determination of superoxide dismutase (SOD) activity

To determine the gastric mucosal activity of SOD, a sample of gastric mucosa was obtained as described above. The colorimetric assay for assessment of SOD activity (Bioxytech SOD-525, Oxis, Portland, USA) has been employed. This method of SOD determination is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. This absorbance was measured by spectrophotometry (Marcel s300, Warsaw, Poland) and the results were expressed as units per gram of gastric tissue (U/g) as reported before (19).

Determination of superoxide dismutase-2 and glutathione peroxidase-1 transcripts by real-time polymerase chain reaction (qPCR)

Expression of mRNA for GPx-1 and SOD-2 in gastric mucosa was determined by real-time PCR as described previously (20, 21). Briefly, RNA was isolated from snap frozen gastric mucosal biopsies using GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland). Reversed transcription to cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Life Technologies, MA, USA). Expression of GPx-1, SOD-2 and β-actin as internal control was determined by real-time PCR using specific primers, SG qPCR Master Mix (2×) including SYBR-Green (EURx, Gdansk, Poland) and appropriate thermal cycler, Quant Studio 12K Flex (Thermo Fisher Scientific, MA, USA). Data was analyzed using the 2-ΔCt method (22, 23).

Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was done using Student's t-test or non-parametric Mann-Whitney U-test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of pretreatment with vehicle or CORM-2 on the macroscopic and microscopic gastric lesions induced by water immersion restraint stress and the alterations in gastric blood flow

Fig. 2A-2D shows the gross macroscopic appearance of the gastric mucosa of rats with intact sensory nerves pretreated with vehicle (control, panel A) or CORM-2 (1 mg/kg i.g., panel B) and exposed 30 min later to 3.5 h of WRS. The representative photomicrographs of gastric mucosa in rats with capsaicin-sensory nerves pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) and exposed 30 min later to 3.5 h of WRS are depicted in *Fig. 2C* and *2D*, respectively. The exposure of vehicle-pretreated rats to WRS resulted in a numerous bleeding erosions mainly observed in the gastric fundic mucosa (*Fig. 2A*) but in CORM-pretreated stomach (*Fig. 2B*) the number of these macroscopic gastric lesions was markedly reduced. In contrast, a distinct exacerbation of gastric lesions induced by WRS as reflected by their increased number was observed in capsaicin-denervated animals pretreated with vehicle and this effect was clearly diminished in those pretreated with CORM-2 (1 mg/kg i.g.) (*Fig. 2C* and *2D*, respectively).

Fig. 3A-3D shows the microscopic appearance of gastric mucosa in rats with intact or sensory denervated rats exposed to 3.5 h of WRS with or without the pretreatment with vehicle (control) or CORM-2 (1 mg/kg i.g.). As depicted in *Fig. 3A* the gastric mucosal damage involving epithelium and connective tissue of oxyntic mucosa was observed in the vehicle-pretreated rats exposed to 3.5 h of WRS. Moreover, the degeneration of the surface epithelium and that of the proximal part of glands as well as the vascular congestion were clearly evident (*Fig. 3A*). In rats pretreated with CORM-2 these histopathological alterations were diminished indicating partial protection of epithelial cellular lining and deeper layers of glandular mucosa showing only mild degeneration (*Fig. 3B*). In capsaicin denervated rats the epithelial cells showed severe damage with distinct signs of desquamation, hemorrhage and cellular white blood cells infiltration (*Fig. 3C*). In contrast, *Fig. 3D* shows that these histopathological alterations in gastric mucosa of capsaicin

denervated rats were improved when these rats have been pretreated with CORM-2 before their subsequent WRS exposure, although the glandular dilatation, vacuolization sometimes vascular congestion were observed within the epithelium at the gastric glands in this experimental group.

The effect of pretreatment with vehicle-control or CORM-2 (1 mg/kg i.g.) on the mean number of gastric lesions as well as accompanying alterations in the GBF are presented in *Fig. 4*. The intact mucosa (control) did not show any grossly visible macroscopic lesions and the GBF in this intact mucosa reached the value of 45 ± 5 ml/min/100 g of the gastric tissue being accepted as the control value (100%) (data not shown). As mentioned above, following 3.5 h of WRS in vehicle-control pretreated rats, the multiple gastric mucosal bleeding erosions were observed mainly in fundic part of gastric mucosa (*Fig. 2A*) and the GBF was significantly reduced comparing with the control value in the intact gastric mucosa ($P < 0.05$, *Fig. 4*). The pretreatment with CORM-2 (1 mg/kg i.g.) in animals with intact sensory nerves, similarly as in our previous study (8, 12), significantly decreased the number of gastric lesions and significantly increased the GBF as compared with the respective values obtained in vehicle-pretreated rats exposed to 3.5 h of WRS ($P < 0.05$, *Fig. 4*). The concurrent treatment with CGRP (10 μg/kg i.p.) with CORM-2 (1 mg/kg i.g.) tended to decrease the number of gastric lesions as compared with rats pretreated with CORM-2 alone but this decrease failed to reach statistical significance (*Fig. 4*). However, such a combination of CGRP with CORM-2 significantly raised the GBF as compared with the respective values in CORM-2-pretreated of animals ($P < 0.05$) (*Fig. 4*). The administration of capsaicin in a dose causing a functional ablation of sensory nerves (9, 12, 13) significantly increased the number of WRS-induced gastric lesions and significantly decreased GBF as compared with respective values obtained in rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$, *Fig. 4*). Similar tendency as in case of animals with intact sensory nerves, was observed in animals with capsaicin-induced denervation because the pretreatment with CORM-2 (1 mg/kg i.g.) significantly decreased the number of gastric lesions and significantly increased GBF as compared with values recorded in vehicle-control pretreated rats with capsaicin denervation, though, this decrease was significantly less pronounced than that in rats with intact sensory nerves pretreated with CORM-2 ($P < 0.05$, *Fig. 4*). The combined administration of CGRP with CORM-2 in capsaicin denervated

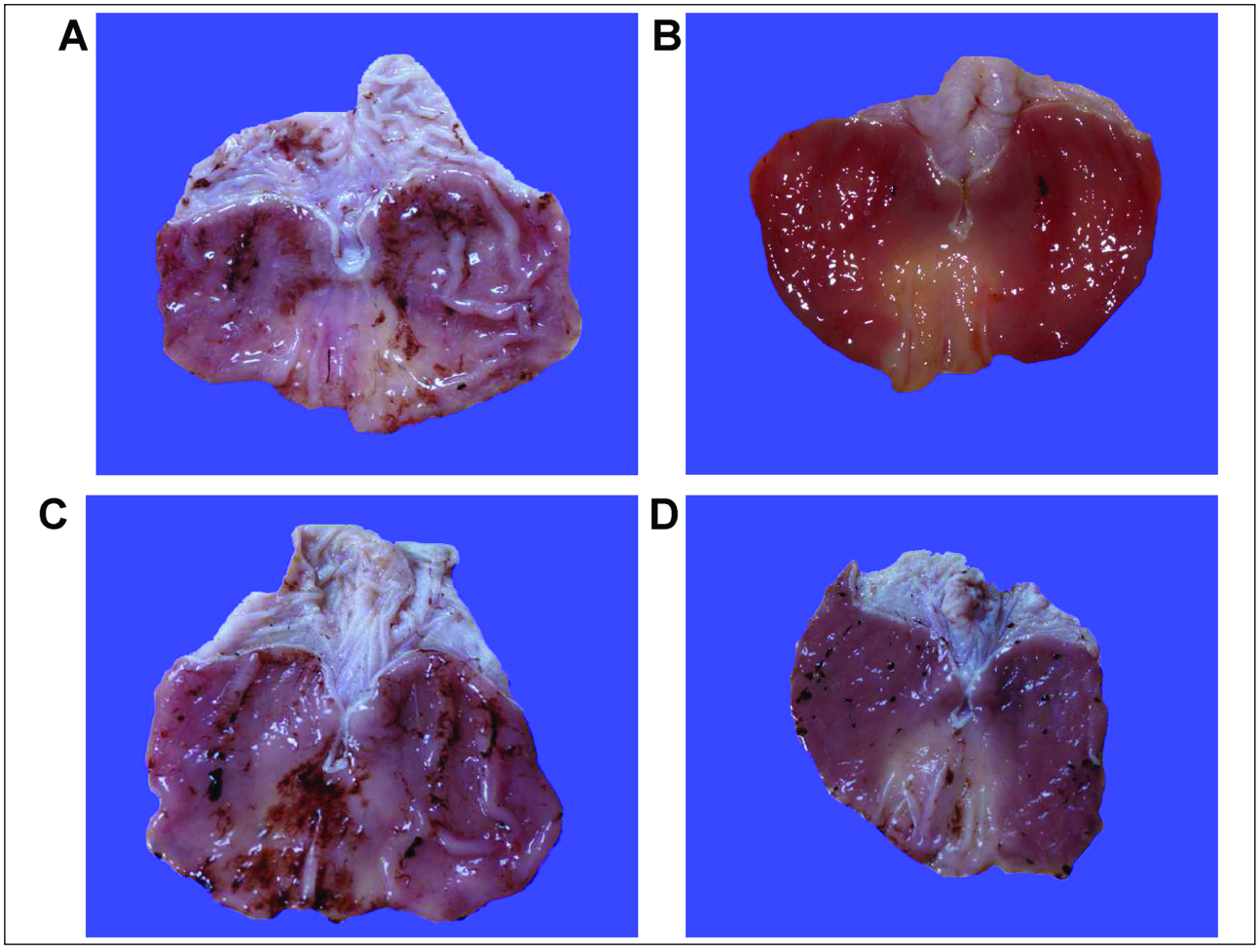

Fig. 2A-2D. The representative appearance of the gastric mucosa of rat pretreated with vehicle (control) and 30 min later exposed to 3.5 h of water immersion and restraint stress (WRS) (*A*) shows the presence of multiple hemorrhagic lesions observed mainly in the oxyntic part of gastric mucosa. In rat pretreated with CORM-2 (1 mg/kg i.g.) and subsequently exposed to WRS, the number of gastric mucosal bleeding erosions is greatly reduced (*B*). The number of WRS-induced gastric lesions is markedly increased in the gastric mucosa of capsaicin-denervated animal (*C*) as compared with that assessed in vehicle-pretreated rat with intact sensory nerves (*A*). The number of WRS-induced gastric mucosal erosions was clearly diminished in the gastric mucosa pretreated with CORM-2 (1 mg/kg i.g.) in rat with capsaicin deactivation of sensory nerves (*D*), though the lesion number was increased in this group of animals when compared with the group of CORM-2-pretreated animals with intact sensory nerves (*B*).

animals also significantly reduced the number of WRS-induced gastric lesions as compared with vehicle-pretreated animals but this alteration in the number of gastric lesions induced by WRS at the combined treatment was found insignificant comparing with the number of gastric lesions achieved in animals pretreated with CORM-2 alone (*Fig. 4*). The similar tendency as in case of rats with intact sensory innervation was however, observed in animals with capsaicin-induced deactivation of sensory nerves regarding the changes in GBF because CGRP co-administered with CORM-2 (1 mg/kg i.g.) significantly increased the GBF in these animals as compared with values recorded in rats pretreated with CORM-2 alone ($P < 0.05$, *Fig. 4*).

Effect of vehicle or CORM-2 on lipid peroxidation in rats exposed to water immersion restraint stress

Fig. 5 shows that the MDA and 4-HNE concentration was significantly increased in vehicle-pretreated rats exposed to WRS as compared with intact gastric mucosa ($P < 0.05$). Pretreatment with CORM-2 (1 mg/kg i.g.) significantly reduced the MDA and 4-HNE concentration as compared to the values measured in gastric mucosa of rats pretreated with vehicle and exposed to 3.5 h of WRS and this effect was further significantly reduced when CGRP was co-administered with CORM-2 ($P < 0.05$, *Fig. 5*). The concentration of MDA and 4-HNE was significantly increased in capsaicin denervated rats above the value recorded in rats with intact sensory nerves exposed to WRS ($P < 0.05$, *Fig. 5*). When CORM-2 (1 mg/kg i.g.) was administered to capsaicin denervated animals, a significant decrease in gastric content of MDA and 4-HNE was observed as compared with vehicle-pretreated capsaicin denervated animals exposed to 3.5 h of WRS ($P < 0.05$, *Fig. 5*). The combination of CGRP (10 µg/kg i.p.) with CORM-2 (1 mg/kg i.g.) significantly decreased the MDA and 4 HNE content as compared with the respective values in CORM-2 pretreated animals with capsaicin-deactivated sensory nerves and exposed to WRS ($P < 0.05$, *Fig. 5*).

Effect of pretreatment with vehicle or CORM-2 on the concentration of glutathione and superoxide dismutase-2 activity in gastric mucosa of water immersion restraint stress exposed rats

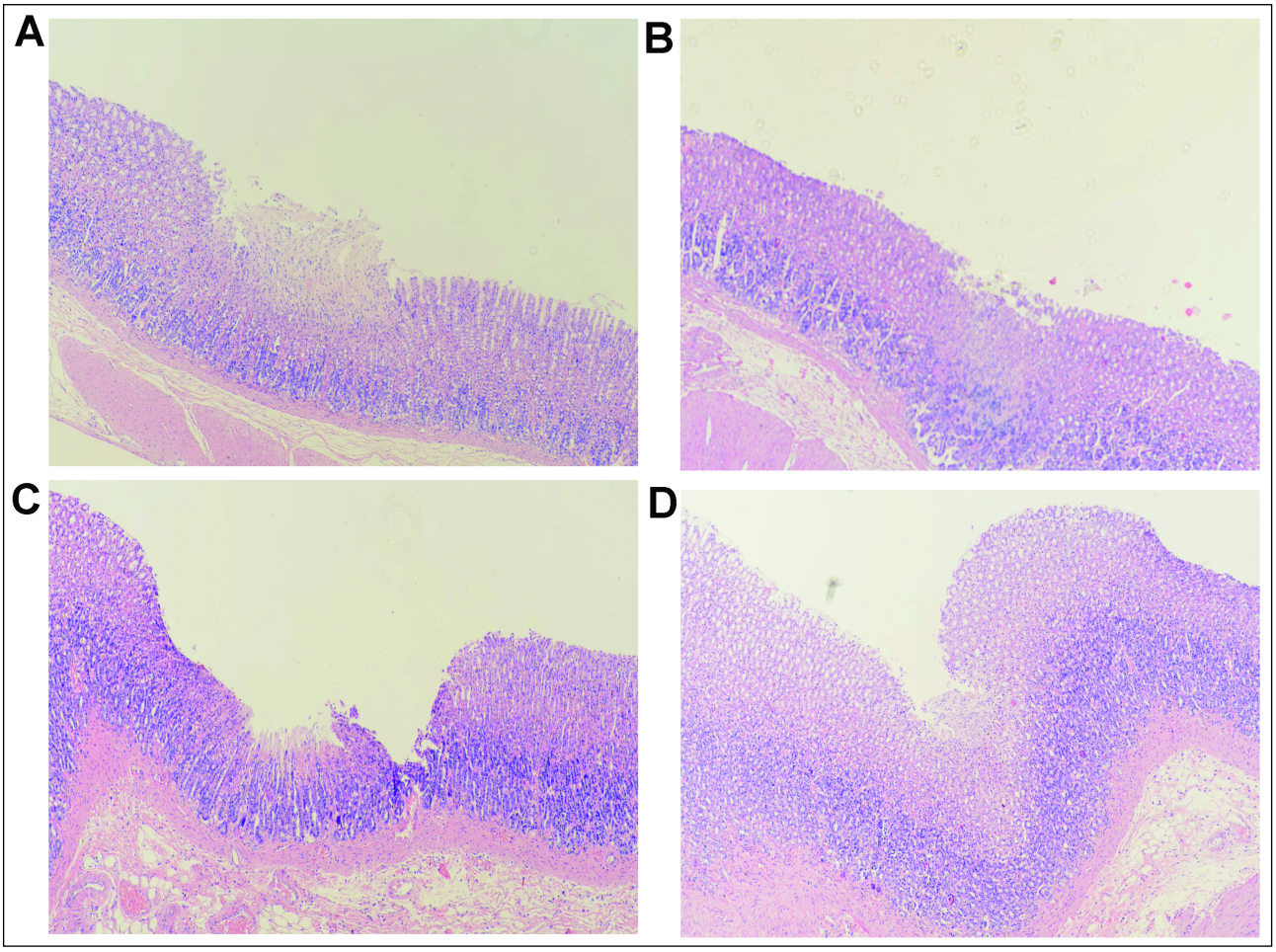

Fig. 3A-3D. The representative microscopic appearance of gastric mucosa in rats with intact or sensory denervated sensory nerves pretreated with vehicle (control) and CORM-2 (1 mg/kg i.g.) and 30 min later exposed to 3.5 h of WRS. In the vehicle-pretreated control rats exposed to WRS, the mucosal damage as reflected by the degeneration of the surface epithelium and connective tissue of oxyntic mucosa as well as the vascular congestion was observed (*A*). In rat pretreated with CORM-2 the epithelial damage was superficial and mild degeneration was observed but epithelial cellular lining and deeper layers of glandular mucosa were diminished indicating partial preservation of mucosal structure (*B*). In capsaicin denervated rat, the epithelial cells showed severe damage with signs of desquamation, hemorrhage and cellular white blood cells infiltration (*C*). These abnormal histopathological pattern observed in gastric mucosa of capsaicin denervated rats pretreated with vehicle and exposed to WRS (*C*) was improved in capsaicin denervated rats pretreated with CORM-2 (1 mg/kg i.g) because partial restoration of glandular structure and less inflammatory exudates were observed despite the glandular dilatation, vacuolization and sometimes vascular congestion observed in these animals (*D*).

with intact sensory nerves and capsaicin-induced sensory denervation

Fig.6 presents the results with the mucosal content of reduced form of glutathione (GSH) in intact gastric mucosa as well as in the gastric mucosa of rats pretreated with CORM-2 with or without the combination with CGRP in WRS-exposed rats with or without capsaicin-denervation. The mucosal concentration of GSH was significantly decreased in gastric mucosa of vehicle-control rats exposed to 3.5 h of WRS, when compared with respective value of GSH measured in the intact mucosa ($P < 0.05$, *Fig. 6*). Pretreatment with CORM-2 applied in a dose 1 mg/kg i.g., significantly increased the GSH content as compared with the value recorded in vehicle-pretreated rats exposed to 3.5 h of WRS ($P < 0.05$, *Fig. 6*). The combined administration of CGRP and CORM-2 significantly increased the GSH concentration in gastric mucosa of stressed animals as compared with that recorded with CORM alone and exposed 30 min later to 3.5 h of WRS ($P < 0.05$, *Fig. 6*). In rats with functional ablation of sensory nerves with capsaicin pretreated with vehicle and exposed to WRS, a significant decrease of GSH concentration was observed as compared with respective values of GSH in vehicle-pretreated rats with intact sensory nerves exposed to WRS ($P < 0.05$, *Fig. 6*). The pretreatment with CORM-2 in capsaicin denervated rats significantly increased the GSH content as compared with vehicle-pretreated rats with capsaicin denervation ($P < 0.05$, *Fig. 6*). When CGRP was administered together with CORM-2 in animals with capsaicin denervation, the mucosal content of GSH was significantly increased over that in vehicle-control and this value reached the level not significantly different observed in rats pretreated with the combination of CGRP and CORM-2 without capsaicin inactivation of sensory nerves ($P < 0.05$, *Fig. 6*).

As shown in *Fig. 7*, the activity of SOD reached the value of 345 ± 14 U/g in intact gastric mucosa but following exposure to WRS in vehicle-pretreated rats, a significant decrease of SOD activity was observed as compared with the respective values in intact gastric mucosa ($P < 0.05$). In rats with intact sensory innervation pretreated with CORM-2 (1 mg/kg i.g.) and exposed to WRS, a significant increase in SOD activity was observed as compared with the respective values in control rats pretreated with vehicle ($P < 0.05$, *Fig. 7*). The concurrent treatment with CGRP (10 µg/kg i.p.) and CORM-2 (1 mg/kg i.g.) failed to affect significantly the gastric mucosal SOD activity above the value observed in CORM-2 alone pretreated animals (*Fig. 7*). The concentration of SOD in capsaicin-denervated animals pretreated with vehicle-control was significantly decreased as compared with respective values obtained in rats with intact sensory nerves pretreated with vehicle and exposed to 3.5 h of WRS ($P < 0.05$, *Fig. 7*). The pretreatment with CORM-2

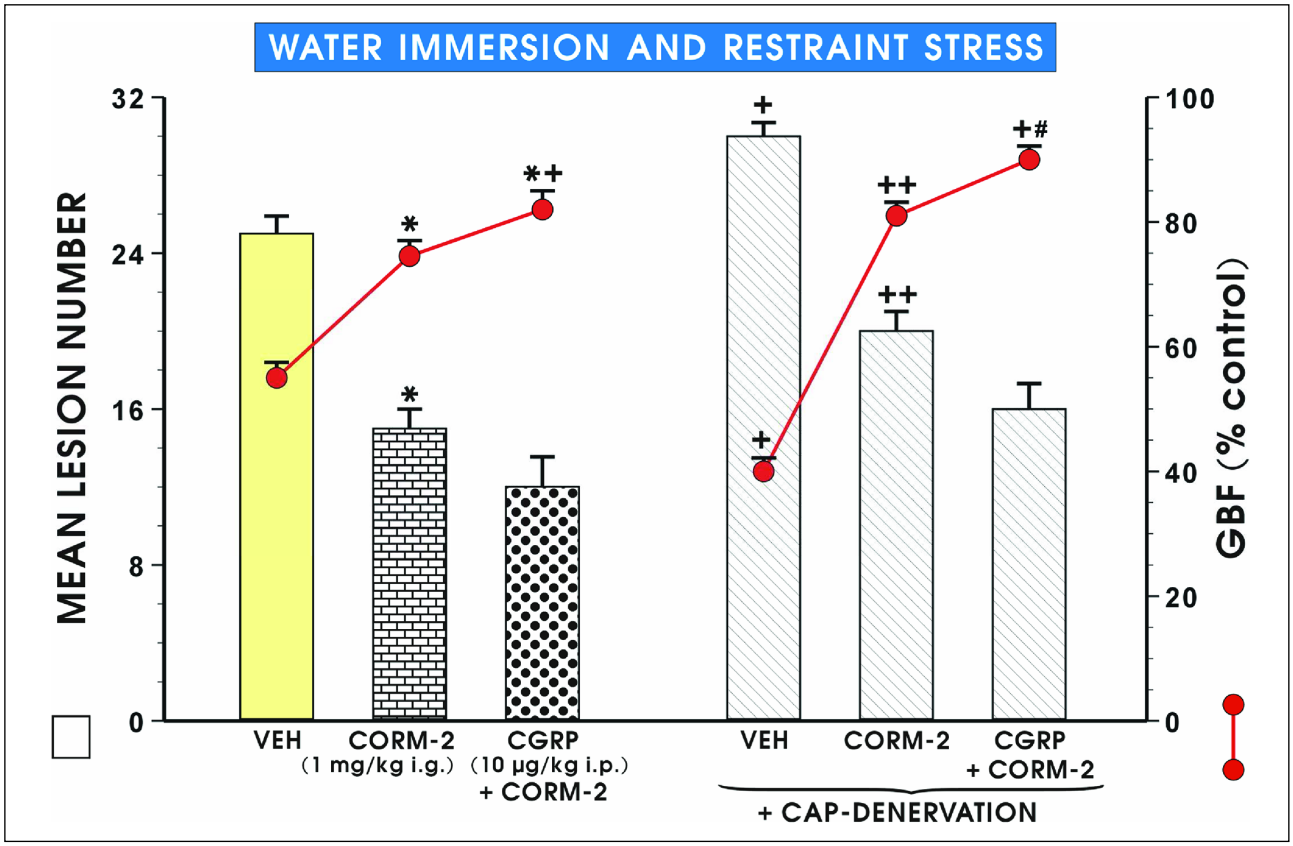

Fig. 4. The mean number of gastric lesions and gastric blood flow (GBF) in rats without or with capsaicin denervation (CAP-DENERVATION) pretreated with CORM-2 (1 mg/kg i.g.) alone or administered in the combination with CGRP (10 µg/kg i.p.) and exposed 30 m later to 3.5 h of water immersion restraint stress (WRS). Results are mean ± S.E.M. of 8 – 10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact (control) group. Asterisk and cross (* +) indicate a significant difference as compared with rats treated with CORM-2 alone ($P < 0.05$). Cross (+) indicates a significant change as compared with the respective values obtained in vehicle-pretreated control rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Double crosses (++) indicate a significant change as compared with the respective values obtained in vehicle-control rats with capsaicin denervation exposed to 3.5 h of WRS ($P < 0.05$). Cross and hash (* #) indicate a significant difference ($P < 0.05$) as compared with the respective values in rats with capsaicin denervation pretreated with CORM-2 alone.

significantly increased the mucosal SOD concentration as compared with vehicle-pretreated rats with capsaicin denervation ($P < 0.05$, *Fig. 7*). The combined administration of CGRP and CORM-2 in animals with capsaicin denervation tended to increase the SOD activity above the value observed in CORM-2 alone pretreated rats with capsaicin denervation but this increase failed to reach statistical significance (*Fig. 7*).

Expression of superoxide dismutase and glutathione peroxidase mRNA in the gastric mucosa of rats with or without capsaicin denervation determined by real time PCR

Figs. 8 and *9* show the data with real time PCR analysis of mRNA expression for GPx-1 and SOD-2, respectively, in the gastric mucosa of intact rats and those with or without capsaicin denervation pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.). The expression of GPx-1 and SOD-2 mRNA was significantly increased in vehicle-pretreated rats exposed to 3.5 h of WRS above that recorded in intact gastric mucosa ($P < 0.05$, *Figs. 8* and *9*). The GPx-1 and SOD-2 mRNA was detectable in the intact gastric mucosa and in those exposed to WRS with or without pretreatment with CORM-2. The analysis of the signal expression revealed that the gastric mucosal expression GPx-1 and SOD-2 mRNA was not significantly affected by CORM-2 in gastric mucosa of rats with intact sensory innervation ($P < 0.05$, *Figs. 8* and *9*). The combined treatment with CGRP and CORM-2 failed to affect significantly the gastric mucosal expression for GPx-1 and SOD-2 mRNA above or below the expression observed in the gastric mucosa of CORM-2 alone pretreated animals exposed to WRS (*Figs. 8* and *9*). In capsaicin denervated animals pretreated with vehicle (control), the significant increase in the expression of GPx-1 and SOD-2 was observed above the respective value recorded in those with intact sensory nerves pretreated with vehicle ($P < 0.05$, *Figs. 8* and *9*). The pretreatment with CORM-2 in rats with capsaicin denervation significantly decreased the expression of GPx-1 and SOD-2 mRNA as compared with vehicle-control pretreated rats with capsaicin denervation ($P < 0.05$, *Figs. 8* and *9*). When capsaicin denervated rats received the combination of CGRP and CORM-2, a significant decrease in the expression of GPx-1mRNA was observed as compared with that detected in CORM-2 alone pretreated rats with capsaicin denervation ($P < 0.05$, *Fig. 8*). The co-treatment of CORM-2 with CGRP tended to

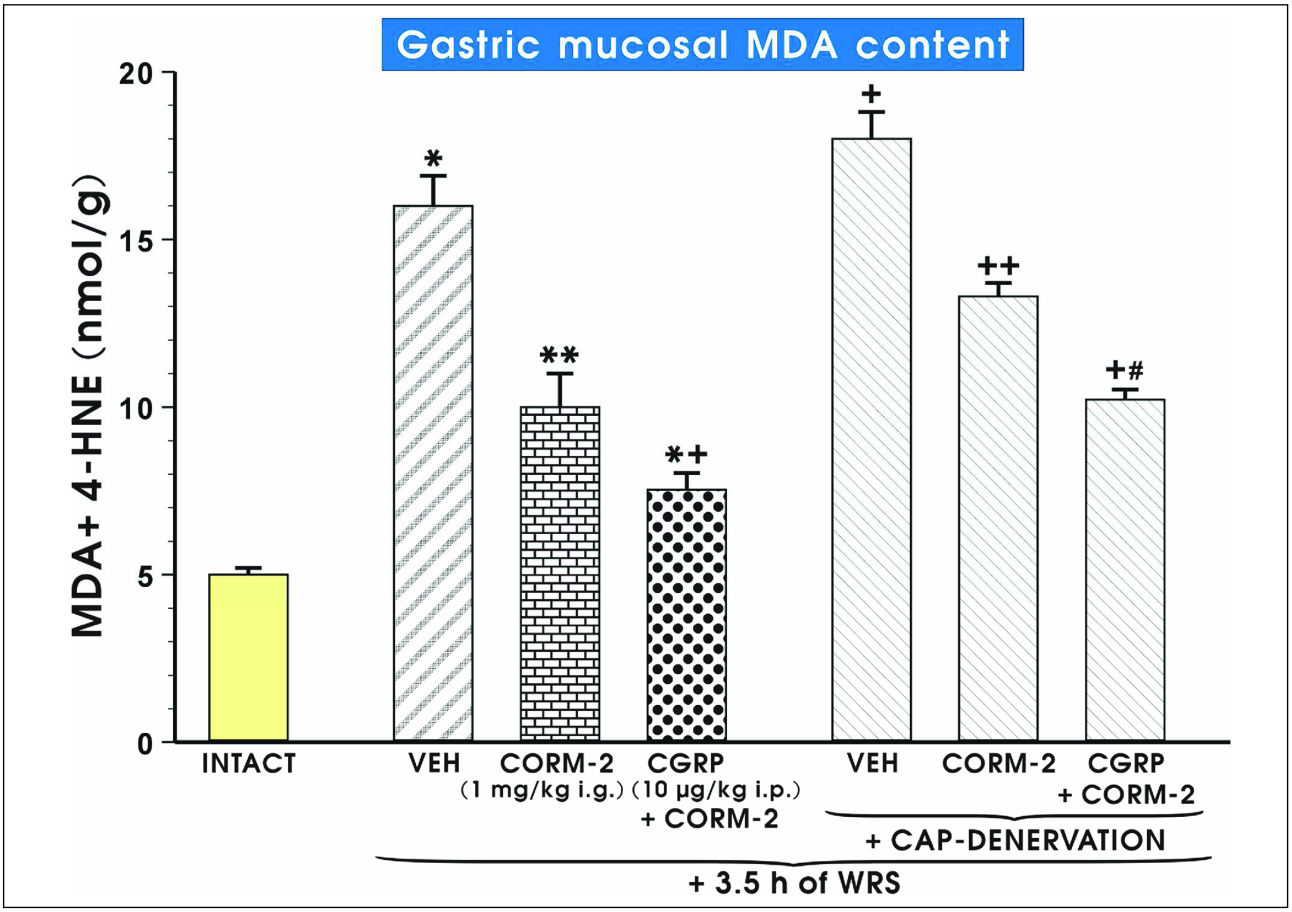

Fig. 5. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats without or with capsaicin denervation (CAP-DENERVATION) pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) or the combination of CORM-2 (1 mg/kg i.g.) with CGRP (10 µg/kg i.p.) and exposed 30 min later to 3.5 h of WRS. Results are mean ± S.E.M. of 8 – 10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Double asterisks (**) indicate a significant change as compared with the respective values obtained in vehicle-pretreated control rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Asterisk and cross (*+) indicate a significant difference as compared with rats with intact sensory nerves pretreated with CORM-2 alone ($P < 0.05$). Cross (+) indicate significant change as compared with the respective values obtained in vehicle-pretreated rats with intact sensory nerves exposed to 3.5 h of WRS alone ($P < 0.05$). Double cross (++) indicate a significant change as compared with the respective values obtained in vehicle-pretreated rats with capsaicin denervation exposed to 3.5 h of WRS ($P < 0.05$). Cross and hash (*#) indicate a significant difference as compared with the respective values in CORM-2 alone pretreated rats with capsaicin denervation ($P < 0.05$).

decrease the expression of SOD-2 mRNA in rats with capsaicin denervation below that recorded with CORM-2 alone but this alteration in mRNA expression of SOD-2 in rats concomitantly treated with CORM-2 and CGRP failed to reach statistical significance (*Fig. 9*).

DISCUSSION

Previous studies documented that reactive oxygen species and the impairment of antioxidative mechanism in gastric mucosa can contribute to the pathogenesis of WRS-induced gastric lesions by induction of acute inflammation and an increase of expression of proinflammatory cytokines including interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) (14-17). These cytokines seem to be responsible for the extravasation of blood vessels and neutrophil infiltration of gastric mucosa under inflammatory conditions (19, 23, 24). It is known that neutrophils exhibit the NADPH oxidase activity, a principal source of a superoxide radical anion ($O_2^{\bullet -}$) (15, 16). We reported that an enhancement of lipid peroxidation and the attenuation of mucosal antioxidative mechanisms, both leading to a decrease in GSH levels by ROS, can contribute to stress ulcerogenesis (14-17, 19, 20).

The present study was designed to determine the potent gastric protective, vasodilatory and anti-oxidative activity of an emerging gaseous mediator carbon monoxide (CO) in the mechanism of gastroprotection against stress-induced gastric damage. First of all, we found that CO released from CORM-2 attenuates gastric lesions induced by WRS and this effect is accompanied by the rise in GBF and the restoration of GSH and SOD activities in rats with intact sensory nerves. On the other hand, the expression of GPx-1 and SOD-2 was upregulated in rats with capsaicin denervation along with the exacerbation of stress-induced gastric lesions observed in these animals. This increased expression of GPx-1 and SOD at the level of mRNA in animals with capsaicin denervation compromised by stress could be explained as compensatory reaction of gastric mucosa to enhance the antioxidative mechanisms in response of capsaicin denervation which rendered this mucosa more susceptible to gastric damage. Moreover, CORM-2 administered alone or in combination with CGRP was highly efficient in protection against WRS damage in rats with or without capsaicin-denervation. Interestingly, the combination of CORM-2 and CGRP tended to

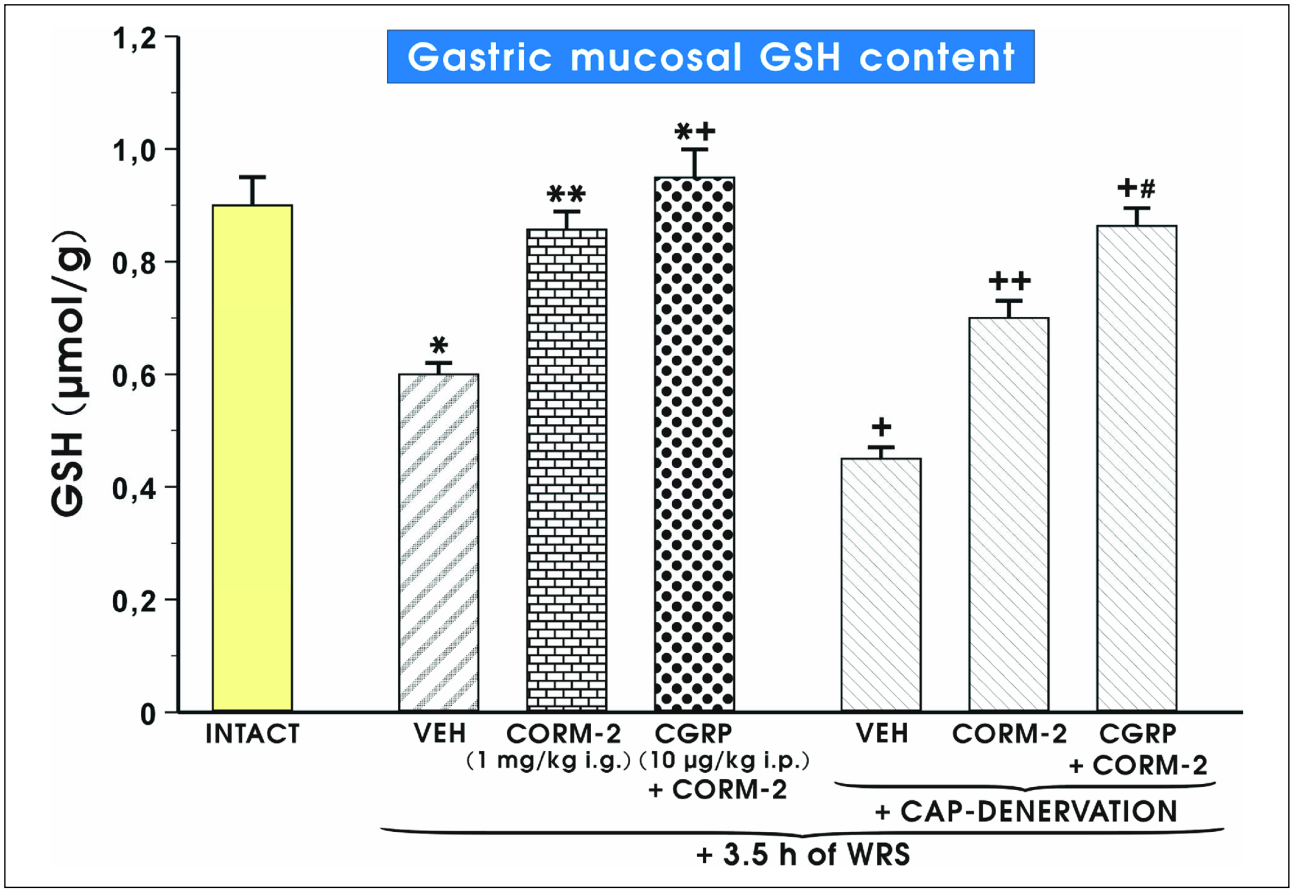

Fig. 6. The concentration of reduced glutathione (GSH) in the gastric mucosa of rats without or with capsaicin denervation (CAP-DENERVATION) pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) or administered with the combination of CORM-2 (1 mg/kg i.g.) and CGRP (10 μg/kg i.p.) and exposed to 3.5 h of WRS. Results are mean ± S.E.M. of 8 – 10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Double asterisks (**) indicate a significant change as compared with the respective values obtained in vehicle-pretreated control rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Asterisk and cross (*+) indicate a significant difference as compared with rats with intact sensory nerves pretreated with CORM-2 alone and exposed to WRS ($P < 0.05$). Cross (+) indicate significant change as compared with the respective values obtained in vehicle-pretreated rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Double crosses (++) indicate a significant change as compared with the respective values obtained in vehicle-pretreated rats with capsaicin denervation exposed to 3.5 h of WRS ($P < 0.05$). Cross and hash (*#) indicate a significant difference as compared with the respective values in CORM-2 alone pretreated rats with capsaicin denervation ($P < 0.05$).

be superior over CORM-2 alone but finally the difference was insignificant indicating that in our experimental model CGRP failed to afford more protection that the pretreatment with CORM-2 applied alone. However, despite the lack of synergistic action of CGRP and CORM-2 to afford greater inhibition of WRS lesions than CORM-2 alone, the significant rise in the GBF was observed after their combined administration in rats with or without capsaicin denervation compared to those pretreated with CORM-2 alone. Moreover, the similar tendency of potentiation of the MDA plus 4HNE and mucosal content of GSH was noticed after the combined administration of CGRP and CORM-2 over those achieved with CORM-2 alone. The reason of this discrepancy between our data with number of gastric lesions which only tended to be decreased after the combined administration of CGRP and CORM-2, with other functional alterations such us GBF and the expression of antioxidizing enzyme GPx-1 mRNA which were synergistically affected by the concurrent treatment with CGRP and CORM-2, at present, remains unknown and should be further determined. However, we can only speculate that the degree of protection with CORM-2 was already maximal and that is why we could not observe the additive effect with exogenous CGRP combined with CORM-2 in protection against stress damage. However, these data does not exclude the possibility that CO can interact with sensory neuropeptide CGRP in the mechanism of protection and gastric hyperemia (25). This study is in keeping with our previous observation that capsaicin denervation resulting in the functional impairment of afferent nerve endings releasing vasoactive neurotransmitter such as CGRP, augmented the gastric mucosal damage induced by the WRS (13-16). Moreover, we provided an evidence that the stress ulcerogenesis, which is accompanied by the increase in the gastric mucosal tissue content of MDA and 4-HNE as well as the prominent fall in SOD and GSH activities, is adversely regulated in rats with capsaicin-induced deactivation of sensory nerves because the MDA and 4-HNE was increased but SOD and GSH activities were decreased under these conditions (22). The donors of CO have recently been shown to increase protective bicarbonate ions secretion in duodenum suggesting that this mechanism may contribute to the gastrointestinal protection (26). Moreover, the inhalation of CO exerted a beneficial effect in diabetic mice presented with gastroparesis, experimental enteritis and protected the intestinal grafts from ischemia-reperfusion injury (27-29). In another studies, CO donors exhibited protection against colitis and ischemic disorders of the liver (30, 31). Moreover, the CO released from CO donors has been shown to afford a sparing protective action against oxidative stress of colon injured by trinitrobenzosulphonic acid (TNBS), the injury to small intestine or the perturbations from thermal stress (32, 33). However, the involvement of the afferent sensory nerves releasing CGRP and lipid peroxidation in the CO-induced protection of

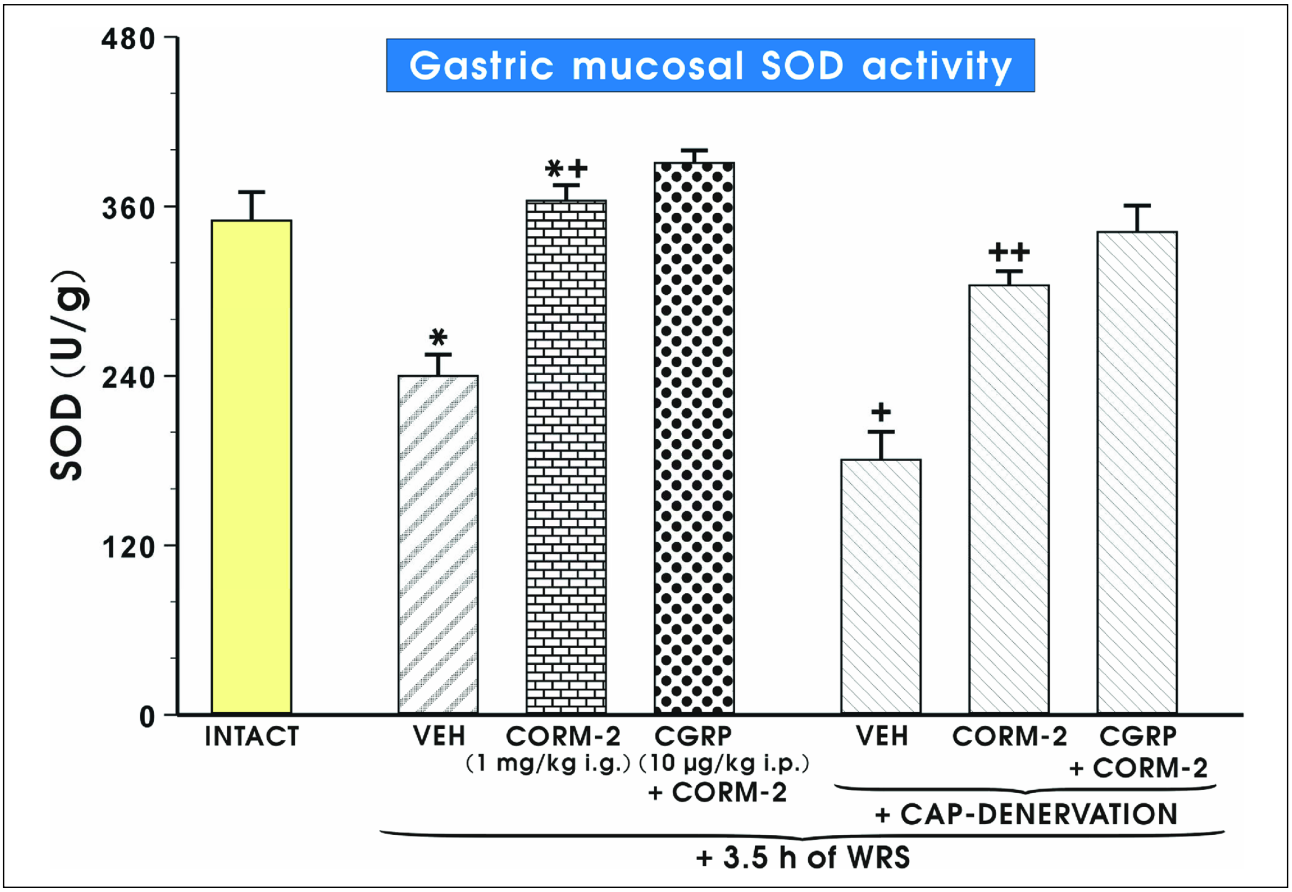

Fig. 7. The superoxide dismutase (SOD) activity in the gastric mucosa of rats with or without capsaicin denervation (CAP-DENERVATION) pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) or administered with the combination of CORM-2 (1 mg/kg i.g.) and CGRP (10 µg/kg i.p.) and exposed 30 min later to 3.5 h of WRS. Results are mean ± S.E.M. of 8 – 10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross (*+) indicate a significant change as compared with the respective values obtained in vehicle-pretreated control rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Cross (+) indicates significant change as compared with the respective values obtained in vehicle-pretreated rats with intact sensory nerves exposed to 3.5 h of WRS alone ($P < 0.05$). Double cross (++) indicate a significant change as compared with the respective values obtained in vehicle-pretreated rats with capsaicin denervation exposed to 3.5 h of WRS ($P < 0.05$).

gastric mucosa compromised by stress conditions have been little studied. We recently reported that CORM-2-releasing CO exhibits a protective and hyperemic effects against gastric lesions induced by WRS (8). Herein, we demonstrated for the first time that this beneficial effect of CORM-2 against stress ulcerogenesis was accompanied by the attenuation of lipid peroxidation as reflected by a decrement of MDA and 4-HNE concentration in the gastric mucosa of rats pretreated with this CO donor. This finding is in keeping with the observation that CO exerted a potent gastroprotective action against ethanol and alendronate-induced gastric damage and prevented small intestine lesions in mice (20, 34-36). We found that CORM-2 not only decreased the MDA content raised in gastric mucosa compromised by stress but also prevented depletion of GSH pool observed in stressed gastric mucosa. This gaseous molecule released from its chemical donors, has been shown to possess the anti-inflammatory properties by decreasing concentrations of proinflammatory cytokines such as IL-1β, IL-8, as well as TNF-β in jejunal and ileal mucosa as well as those in the liver (29, 32, 33, 37).

High amounts of nitric oxide (NO), generated during inflammation are considered as the source of peroxynitrite ($ONOO^-$) wich accelerated oxidative tissue damage (38). Interestingly, CO exerted protective effect on small intestine mucosal cells through inhibition of NO generation and attenuation of expression of inducible NO synthase (iNOS) in intestinal tissue damaged by $ONOO^-$ from enhanced iNOS expression (32). Administration of CO donor resulted in inhibition of inflammatory process, infiltration of tissue by neutrophils, expressed by decrease of myeloperoxidase (MPO) activity and concentrations of proinflammatory cytokines, such as TNF-α and IL-1β (26). Nakao *et al.* have shown immunomodulative effect of CO inhalation resulting in attenuation of ischemic damage of small intestine in rats (28). The mechanism of this phenomenon in their study (28) was possibly related to conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). Inhaled CO at the concentration of 225 ppm per hour, administered 1 h before and 24 h after reperfusion, reduced intestinal damage by mechanism involving an increase of intestinal microcirculation mediated by cGMP, the inhibition of proinflammatory cytokines TNF-α, IL-1β and endothelial cells apoptosis (28). This beneficial action of CO was extended to other organs because CO prevented rat liver damage induced by ischemia and reperfusion (31). It is of interest that CO released from CORM-2 attenuated the ischemic liver damage resulting from the generation of ROS during reperfusion that caused the oxidative modification of lipids and proteins, the induction of apoptosis in hepatocytes as well as an increase in activity of proinflammatory cytokines (31). Ibrahim *et al.* confirmed that

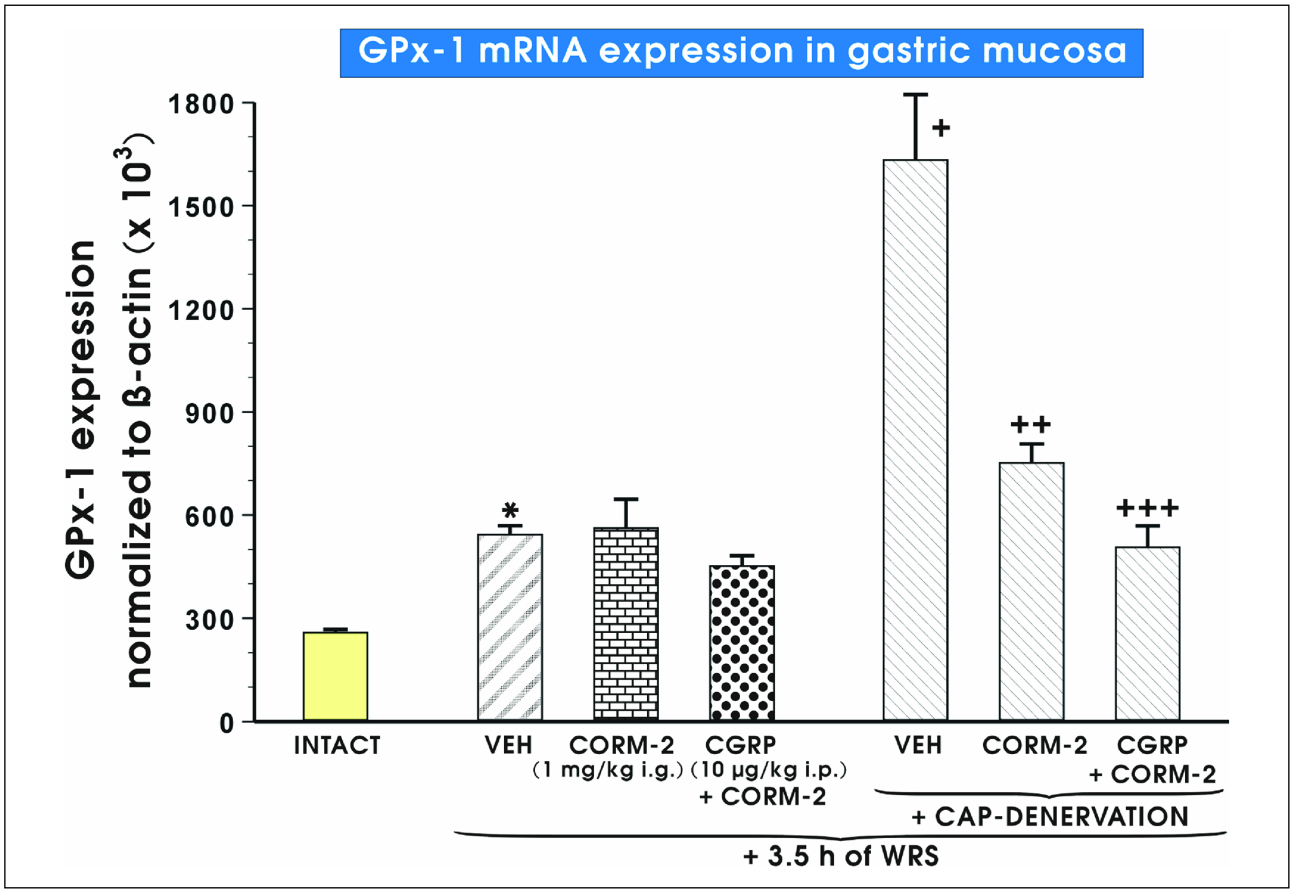

Fig. 8. The messenger RNA expression for glutathione peroxidase (GPx) analyzed by real-time PCR in the intact gastric mucosa and in rats with or without capsaicin denervation (CAP-DENERVATION) pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) or administered with the combination of CORM-2 (1 mg/kg i.g.) and CGRP (10 µg/kg i.p.) and exposed 30 min later to 3.5 h of WRS. Asterisk (*) indicates a significant change as compared to the value obtained in intact gastric mucosa ($P < 0.05$). Cross (+) indicates significant change as compared with the respective values obtained in vehicle-pretreated rats with intact sensory nerves exposed to 3.5 h of WRS ($P < 0.05$). Double crosses (++) indicate a significant change as compared with the respective values obtained in vehicle-pretreated rats with capsaicin denervation exposed to 3.5 h of WRS ($P < 0.05$). Triple crosses indicate a significant difference as compared with the respective values in group of capsaicin-denervated rats pretreated with CORM-2 alone and exposed 30 min later to WRS ($P < 0.05$).

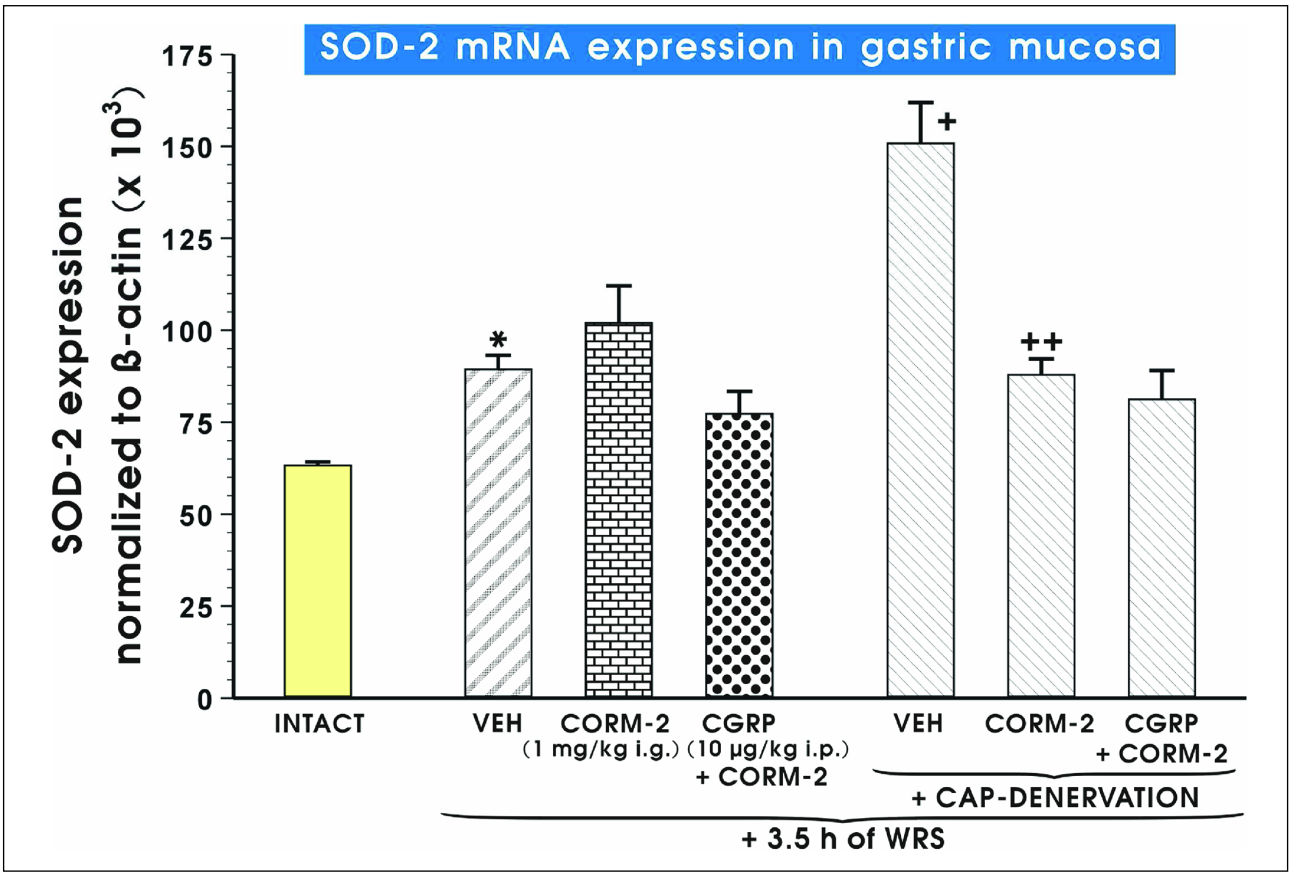

Fig. 9. The messenger RNA expression for superoxide dismutase (SOD) analyzed by real-time PCR in the intact gastric mucosa and in rats with or without capsaicin denervation (CAP-DENERVATION) pretreated with vehicle (control) or CORM-2 (1 mg/kg i.g.) or administered with the combination of CORM-2 (1 mg/kg i.g.) and CGRP (10 µg/kg i.p.) and exposed 30 min later to 3.5 h of WRS. Asterisk (*) indicates a significant change as compared to the value obtained in intact gastric mucosa ($P < 0.05$). Cross (+) indicates a significant change as compared with the value obtained in vehicle-pretreated rats exposed to 3.5 h of WRS ($P < 0.05$). Double crosses (++) indicate a significant change as compared with the respective values obtained in vehicle-pretreated rats with capsaicin deactivated sensory nerves exposed to 3.5 h of WRS ($P < 0.05$).

endogenous CO produced by HO-1 afforded protection against cold restraint stress-induced gastric lesions in mice and these effects were accompanied by decrease of lipid peroxidation (39). De Backer and Lefebvre (40) revealed an important interaction between endogenous CO and SOD activity in maintenance of physiological contractile functions of gastric fundus and jejunum in mice exposed to oxidative stress. Finally, newborn mice deficient of HO-1 enzyme developed necrotizing colitis supporting the importance of HO-1/CO pathway in the development of intestinal integrity and mucosal defense (41).

In conclusion, an intragastric pretreatment with CORM-2 releasing CO exerts gastroprotective effect against stress-induced gastric lesions via mechanism involving an increase in gastric microcirculation, the decrease of MDA+4-HNE content considered as an index of lipid peroxidation, the activation of antioxidative defense mechanisms manifested by restoration of the activity of gastric mucosal SOD and GSH levels decreased in stressed gastric mucosa and the attenuation of expression of SOD-2 and GPx-1 mRNA expression markedly increased in the gastric mucosa compromised by stress. The functional ablation of sensory nerves by capsaicin exacerbated WRS-induced gastric damage and raised the expression of SOD-2 and GPx-1 mRNA in stressed gastric mucosa but CORM-2 attenuated these lesions and produced an increase in the GBF, the mucosal content of SOD and GSH and normalized the expression of SOD-2 and GPx-1 mRNA. Furthermore, our study provides an evidence that CO can attenuate the process of lipid peroxidation and counteracts the failure of antioxidative enzyme activity of GPx and SOD-2 and reduces the rise in expression of mRNA for GPx-1 and SOD under stress conditions. Thus, our data does not exclude the possibility that sensory nerve releasing vasoactive neuropeptides such as CGRP are involved in CO-induced gastroprotection against stress ulcerogenesis but this issue requires the confirmation in the future studies.

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