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HYDROGEN SULPHIDE PREVENTS ETHANOL-INDUCED GASTRIC DAMAGE IN MICE: ROLE OF K_{ATP} CHANNELS AND CAPSAICIN-SENSITIVE PRIMARY AFFERENT NEURONS.

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List of Abbreviations:

K_{ATP}, ATP-sensitive potassium channels; GSH, reduced glutathione; MDA, malondialdehyde; CSE, cystathionine γ-lyase; CBS, cystathionine β-synthetase.

- Recommended sections: Gastrointestinal, Hepatic, Pulmonary, and Renal.

Abstract:

The aim of this study was to evaluate the protective effect of H_2S on ethanol-induced gastric lesions in mice and the influence of K_{ATP} channels, capsaicin-sensitive sensory afferent neurons and TRPV1 receptors on such effect. Saline and L- cysteine alone or with propargylglycine, NaHS or Lawesson´s reagent were administrated for testing purposes. For other experiments, mice were pre-treated with glibenclamide, neurotoxic doses of capsaicin or capsazepine. Afterwards, mice received L- cysteine, NaHS or Lawesson´s reagent. After 30 min, ethanol 50% was administrated by gavage. After 1 hour, mice were sacrificed and gastric damage was evaluated by macroscopic and microscopic analyses. L-cysteine, NaHS and Lawesson´s reagent treatment prevented ethanol-induced macroscopic and microscopic gastric damage in a dose-dependent manner. Administration of propargylglycine, an inhibitor of endogenous H_2S synthesis, reversed gastric protection induced by L-cysteine. Glibenclamide reversed L-cysteine, NaHS or Lawesson´s reagent gastroprotective effects against ethanol-induced macroscopic damage in a dose dependent manner. Chemical ablation of sensory afferent neurons by capsaicin reversed gastroprotective effects of L- cysteine or H_2S donors (NaHS or Lawesson´s reagent) in ethanol-induced macroscopic gastric damage. Likewise, in the presence of the TRPV1 antagonist capsazepine, the gastroprotective effects of L- cysteine, NaHS or Lawesson´s reagent were also abolished. Our results suggest that H_2S prevents ethanol-induced gastric damage. Although there are many mechanisms through which this effect can occur, our data supports the hypothesis that the activation of K_{ATP} channels and afferent neurons/TRPV1 receptors are of primary importance.

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Introduction

Ethanol promotes the rapid formation of ulcers in the stomach, which occurs mainly due to an inflammatory reaction (Szabo et al, 1985). Ethanol-induced gastric damage is characterized by epithelial cellular loss, mucosal oedema and sub- epithelial hemorrhage (Guslandi, 1987; or Medeiros et al., 2008).

Hydrogen sulphide (H_2S) is generally known as a toxic (in high concentration) and colorless gas with a strong odor, produced from a variety of environments but is also found in mammalian tissues, where it is generated during cysteine metabolism (Guidotti, 1996). H_2S is formed in mammalian cells by the activity of two pyridoxal phosphat-dependent enzymes: cystathionine γ-lyase (CSE) and cystathionine β-synthetase (CBS) (Moore et al., 2003). Similarly to other gaseous mediators (nitric oxide and carbon monoxide), H_2S seems to present a paradoxical effects in the inflammatory process: there are evidences that it is involved both as an agent preventing tissue damage, leukocyte migration, oedema formation and nociception and, as an agent causing tissue damage, leukocyte migration and oedema (Fiorucci et al., 2006; Li et al., 2006; or Cunha et al., 2008).

Gastric mucosa expresses both CSE and CBS, which have the ability to mediate H_2S synthesis (Fiorucci et al., 2005). In the gastrointestinal tract (GI), recent studies suggest that H_2S may contribute to mucosal defense against injury caused by anti-inflammatory nonsteroidal drugs (NSAIDs) and it also seems to play a significant role in regulating gastric mucosal blood flow. The mechanisms through which H_2S exerts these anti-inflammatory properties are not fully understood. However, the involvement of K_{ATP} channels might be considered (Fiorucci et al., 2006), since, it was already demonstrated that H_2S reduces leukocyte infiltration and oedema formation via K_{ATP} channel activation (Zanardo et al., 2006). On the other hand, Wallace et al. (2007) demonstrated that H_2S promotes healing of gastric ulcers in rats by a mechanism that is

not associated with K_{ATP} channels, because neither glibenclamide (K_{ATP} channel antagonist) nor pinacidil (K_{ATP} channel activator) affected ulcer healing.

The maintenance of GI mucosal integrity depends on protective mechanisms in the face of pending injury. Capsaicin-sensitive sensory afferent fibers subserve this goal through different mechanisms. The protective responses triggered by sensory neurons comprise alterations in GI blood flow, secretion, and motility as well as modifications of the immune function (Holzer, 2007; or Szolcsanyi and Bartho, 2001). However, there are only a few correlative reports between H_2S and sensitive sensory afferent neurons and the involvement of the former upon GI functions. Patacchini et al (2004) demonstrated that H_2S stimulates capsaicin-sensitive sensory afferent fibers in the rat urinary bladder and Trevisani et al (2005) pointed to a role of H_2S in the activation of tachykinin-induced neurogenic inflammatory responses in guinea-pig airways, and that this effect is mediated by stimulation of TRPV1 receptors. Moreover, Shicho et al., 2006 demonstrated that H_2S also stimulated enteric neurons via TRPV1 receptor on extrinsic afferent terminals.

Considering the little information concerning the pathophysiological role of H_2S on gastric damage, we evaluated the protective effect of H_2S on ethanol-induced gastric lesions in mice and the influence of K_{ATP} channels and capsaicin-sensitive sensory afferent neurons and TRPV1 receptors on this effect.

Methods

Animals

Male Swiss mice (weight 25-30g) were fasted 18-24 hours before the experiments. Animals were housed in cages in temperature-controlled rooms and received water and food *ad libitum*. All animal treatments and surgical procedures were performed in accordance to the

Guide for Care and Use of Laboratory Animals, National Institute of Health (Bethesda, MD, USA) and were approved by the local ethics committee (protocol N^{o} 63/07).

Drugs

L-cysteine, DL-propargylglycine (PAG), capsazepine, capsaicin and glibenclamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lawesson's reagent was obtained from Fluka (India). Vehicle solutions consisted of Tris (Merck, São Paulo, SP, Brazil) buffer or saline. Glibenclamide was dissolved in 0.01N NaOH containing 4% glucose.

H_2S donors in ethanol-induced gastric damage

Mice were treated by gavage with saline, cysteine (H_2S precursor, 25, 50 and 100 mg kg^{-1}), NaHS (H_2S donor, 75, 150 and 300 μmol kg^{-1}) or Lawesson's reagent (H_2S donor, 3, 9, 27 and 81 μmol kg^{-1}). Another group received DL-propargylglycine (an inhibitor of the CSE, 15, 50 and 150 mg kg-1, p.o). After thirty minutes, ethanol 50% (0.5 ml 25g^{-1}) was administered by gavage to all groups, as adapted from Robert, 1979. The control group received only saline or saline + ethanol. Additional experiment using Lawesson's reagent at the dose of 27 μmol kg^{-1}was made. After thirty minutes, six hours or twenty four hours, ethanol 50% (0.5 ml 25g^{-1}) was administered by gavage. One hour later, animals were sacrificed and their stomachs rapidly removed, opened through an incision along the greater curvature and pinned out on a wax block. Hemorrhagic or ulcerative lesions were measured using a computer planimetry program (Image J). A sample of the corpus region of each stomach was fixed in 10% formalin for subsequent histopathological assessment. Other full-thickness pieces of the gastric corpus were then weighed, frozen and stored at 70^{o}C until assayed for glutathione (Sedlak and Lindsay, 1968) or malondialdehyde **(**MDA) (Mihara and Uchiyama (1978).

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ATP-sensitive potassium channels (K_{ATP}) in H_2S donors protective effect

In order to study the role of K_{ATP} in H_2S donors protective effect, mice were treated with glibenclamide (3 or 10 mg kg^{-1}, i.p.). After one hour, mice received NaHS (150 μmol kg^{-1}) by gavage or Lawesson´s reagent (27 μmol kg^{-1}). Thirty minutes later, gastric damage was induced by intragastric instillation of ethanol 50% (0.5 ml $25g^{-1}$ by gavage). The control group received only saline or NaOH 0.01N containing 4% glucose. The control group was also administered glibenclamide (10 mg kg^{-1}, i.p) without H_2S donor and before absolute ethanol administration. One hour later, gastric damage was determined as described above. Finally, a sample of the corpus region of each stomach was fixed in 10% formalin for subsequent histopathological assessment, and other full-thickness pieces of the gastric corpus were weighed, frozen and stored at -70° C for glutathione or MDA assays.

Chemical ablation of sensory afferent neurons by capsaicin and role of TRPV1 receptor

To evaluate the involvement of sensory afferent neurons in the gastroprotective effectsof NaHS and Lawessen's reagent, mice were injected with capsaicin (25 mg kg^{-1} plus 2 x 50 mg kg^{-1}, s.c., with an interval of 12h between each dose) for chemical ablation of C fibers, as described by Maggi et al., (1988) or Ehrlich et al., (2004) and adapted to our experimental conditions. To counteract any respiratory impairment, animals were anesthetized with tribromoethanol solution 2,5% (250 mg kg^{-1}, i.p.) and given theophylline (20 mg kg^{-1}, i.m.), before capsaicin administration. After 8 days, one or two drops of capsaicin solution (10 μg ml^{-1}) were instilled in the ocular globe, monitoring the wiping reflex to find out if animals had been desensitized. The absence of this reflex was taken as an effective ablation of primary afferent sensory neurons. Two groups (n = 8), of naïve mice were treated with vehicle or NaHS (150 μmol kg^{-1}, p.o.) or Lawessen's reagent (27 μmol kg^{-1}, p.o.) as previously described before the injection of ethanol

50% by gavage. Similar treatments were applied to two other groups of mice that were capsaicin-desensitized. In addition, a saline-treated group (normal control) was included. In another experimental setting, mice were treated with capsazepine (TRPV1 receptor antagonist, 5 mg kg^{-1}, i.p.) 30 min prior to NaHS (150 μmol kg^{-1}, p.o.) or Lawessen's reagent (27 μmol kg^{-1}, p.o.) treatment (Campos et al., 2008). Ethanol 50% (0.5 ml $25g^{-1}$, p.o.) was then injected 30min after H_2S donors. In all protocols, treatment effects on gastric lesions were verified as previously described.

Histological assessment

For histological assessment, the glandular stomach was fixed in 10% neutral buffered formalin solution, sectioned and embedded in paraffin. Four micrometer-thick sections were deparaffinized, stained with hematoxylin and eosin, and then examined under a light microscope. Specimens were assessed according to the criteria of Laine et al*.,* 1988. In brief, a 1-cm length of each histological section was assessed for epithelial cell loss (a score of 0 to 3), oedema in the upper mucosa (a score of 0 to 4), hemorrhagic damage (a score of 0 to 4), and presence of inflammatory cells (a score of 0 to 3). Afterwards, sections were blinded evaluated by an experienced pathologist (GACB).

GSH assay

Reduced glutathione (GSH) content in stomach tissues as non-protein sulfhydryls was estimated according to the method described by Sedlak and Lindsay (1968). A glandular segment from each stomach was homogenized in 5ml of ice-cold 0.02M EDTA solution (1 ml 100 mg^{-1} tissue). Aliquots (400 μl) of tissue homogenate were mixed with 320 μl of distilled water and 80 μl of 50% (w/v) trichloroacetic acid in glass tubes and centrifuged at 3000 x *g* for 15 min.

Supernatants (400 µl) were mixed with 800 µl Tris buffer (0.4 M, pH 8.9), and 20 µl 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB; 0.01 M) were added. After shaking the reaction mixture, absorbance was measured at 412 nm within 5 min of DTNB addition against a blank with no homogenate. Glutathione concentration was read off a standard curve and expressed as µg GSH g^{-1} of wet tissue.

MDA assay

The level of MDA in the homogenate from each group was measured using the method of Mihara and Uchiyama (1978).

Determination of malondialdehyde precursor in tissues by thiobarbituric acid test: briefly, 250 μl of 10% homogenate of the tissue sample, add 1,5 ml of 1% H_3PO_4 and 0,5 ml of 0.6% TBA aqueous solution; stir and heat the mixture on a boiling water bath for 45 min. After cooling, add 2 ml of *n*-butanol, shake, and separate the butanol layer by centrifugation; determine optical density of butanol layer at 535 and 520 nm and calculate optical density difference between the two determinations to be taken as the TBA value. MDA concentrations are expressed as nanomoles per gram of tissue.

Statistical analysis

All values are expressed as means ± S.E.M. ANOVA and Student–Newman–Keuls test were used to determine statistical significance of differences between groups. For histological assessment, the Kruskal-Wallis nonparametric test was used, followed by Dunn's test for multiple comparisons. Differences were considered to be significant when $P \leq 0.05$.

Results

In Figure 1, we observed that L-cysteine (panel *A*), NaHS (panel *B*) or Lawesson´s reagent (panel *C*) treatment prevented ethanol-induced macroscopic gastric damage in a dose-dependent manner. Administration of propargylglycine, an H_2S synthesis inhibitor (PAG) that blocks CSE activity, reversed gastric protection induced by cysteine in ethanol-induced gastric damage (Figure 1, panel *A*). We also observed that Lawesson´s reagent at the dose of 27 μmol kg^{-1} prevented ethanol-induced macroscopic gastric damage in a time-dependent manner, with greatest effect after 30 minutes (45.66 ± 7.11 mm^2), which persist after 6 hours (51.19 ± 7.51 mm^2), but was a complete reversal 24 h after the Lawesson's reagent treatment (90.19 ± 8.19 mm^2).

In the microscopic analysis, NaHS and Lawesson´s reagent decreased hemorrhagic damage, oedema and epithelial cells loss induced by ethanol. L-cysteine treatment reduced mainly hemorrhagic damage (Table 1). Figure 2 showed that ethanol administration induced disruption of the gastric gland superficial region with epithelial cell loss and intense hemorrhage (panel *B*). We did not observe these alterations in mice pretreated with L-cysteine (panel *C*), NaHS (panel *D*) or Lawesson´s reagent (panel *E*). However, the L-cysteine gastroprotective effect was abolished when the mice were pretreated with PAG (panel *F* and table 1).

The administration of ethanol reduced and increased the gastric GSH and MDA concentrations, respectively. The L- cysteine and H_2S donors (NaHS or Lawesson´s reagent) pre-treatments prevented these effects of ethanol. Administration of PAG, reversed the L- cysteine-gastroprotective effects on ethanol-induced gastropathy in mice (table 2).

To assess the contribution of K_{ATP} channels in the protective effects of L- cysteine or H_2S donors, mice were pretreated with glibenclamide. The glibenclamide pretreatment prevented in a

dose-dependent manner the protective effects of L-cysteine (panel A), NaHS (panel B) or Lawesson´s reagent (panel C) against ethanol-induced macroscopic damage (figure 3).

Chemical ablation of sensory afferent neurons by capsaicin treatment prevented the gastroprotective effects of L- cysteine or H_2S donors (NaHS or Lawesson´s reagent) on ethanol-induced-macroscopic gastric damage (Figure 4). Likewise, the pretreatment of the mice with capsazepine, a TRPV1 antagonist, abolished the gastroprotective effects of L- cysteine, NaHS or Lawesson´s reagent on ethanol-induced gastric lesions (Figure 5).

Discussion

While long recognized as an industrial pollutant, hydrogen sulphide is increasingly identified as an important mediator of many physiological processes. H_2S has been proved to be a neuromodulator (Wang, 2002), an endogenous regulator of acute inflammation (Zanardo et al., 2006; or Dal Secco et al., 2008) and pain (Distrutti et al., 2005; or Cunha et al., 2008). It was also demonstrated that H_2S contributes to the maintenance of gastric mucosal integrity in damage caused by anti-inflammatory nonsteroidal drugs (Fiorucci, 2005). In the present study, we evaluated the protective effect of hydrogen sulphide against ethanol-induced gastric damage in mice and the role of K_{ATP} channels and capsaicin-sensitive sensory afferent fibers.

Our results confirmed that ethanol administration at high concentrations caused severe macroscopic and microscopic gastric mucosal damage, with hemorrhage, oedema and epithelial cell loss (Guslandi, 1987; Laine and Weinstein, 1988; or Medeiros et al., 2008). In the present study, we observed that both H_2S donor and H_2S precursor (L- cysteine) decreased ethanol-induced gastropathy. This effect was greatest after 30 minutes, persist for 6 hours, but was reversed 24 h after the H_2S donor treatment. Furthermore, propargylglycine (an irreversible inhibitor of CSE) pretreatment prevented the protective effect of L-cysteine on ethanol-induced

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gastric damage. These findings are consistent with the hypothesis that gastroprotective effects of L-cysteine were correlated to increased H_2S synthesis. Therefore, we could infer that H_2S synthesis is essential to gastric protection against ethanol.

It was described that the pathophysiology of ethanol-induced gastric mucosal injury is related to the release of inflammatory mediators, which induce activation of granulocytes with proteases/free radical formation, and a decrease in gastric blood flow that provoke gastric mucosal ischemia, cell death and gastric damage (Teyssen and Singer, 2003). The role of H_2S on leukocyte migration is controversial. There are evidences showing that it inhibits leukocyte-endothelial cell adhesion, (Fiorucci et al., 2006; or Zanardo et al., 2006) and, on contrary, evidences that it may enhances the neutrophil migration (Dal Secco et al., 2008). The effect of H_2S upon leukocyte migration does not elucidate the H_2S gastroprotective effects in our models, since we did not observe an increase in inflammatory cell infiltration after ethanol administration. Thus, it possible that the H_2S gastroprotective effect may be related to an increase in gastric blood flow, as it was demonstrated that H_2S enhanced gastric blood flow in the stomach during NSAID- induced gastropathy (Fiorucci et al., 2005).

It is also possible that the effect of H_2S may decrease redox state in ethanol induced gastropathy. Our results showed that administration of L-cysteine and H_2S donors reversed the decrease in gastric GSH level after ethanol administration. Indeed, there is an association between glutathione and H_2S synthesis considering that L-cysteine is a common substrate for both processes (Wallace et al., 2007). We could infer that the protective effect of L- cysteine might be explained by an increase in GSH concentration. However, Wallace et al. demonstrated that the administration of L-cysteine did not increase glutathione levels in the gastric mucosa (Wallace et al., 2007). Therefore, another possibility is that an increase in GSH levels could be secondary to a decrease in free radical production. Our results demonstrated that L-cysteine or

H_2S donors administration resulted in a significant decrease in MDA concentrations in ethanol-induced gastropathy. Thus, the mechanism through which H_2S exerts its gastroprotective effect seems to involve the reduction of lipid peroxidation induced by ethanol instillation.

Next, we investigated the possible role of K_{ATP} channels in H_2S donors gastroprotective effects on ethanol-induced gastric damage. Many effects of H_2S, including vasodilation, inhibition of leukocyte adherence (Fiorucci et al., 2006), analgesic effect (Distrutti et al., 2006) and inhibition of oedema formation, have been found to be inhibited by glibenclamide, a K_{ATP} channel antagonist. In our results, we have shown that glibenclamide reversed both L- cysteine and H_2S donors protective effect against ethanol-induced gastric damage during the lesion d development. Fiorucci et al. also observed that inhibition of H_2S effect by glibenclamide contributes to an increase in NSAIDs-induced gastric damage (Fiorucci et al., 2005), suggesting an involvement of K_{ATP} channels in H_2S gastroprotective effects. On the other hand, Wallace et al. (2007) demonstrated that K_{ATP} channels activation by pinacidil did not produce any beneficial effects on ulcer healing as observed in the case of H_2S donors. Moreover, glibenclamide did not affect ulcer healing. So these authors shows that H_2S makes a significant role to the healing of ulcers and these effects may be related to the vasodilator actions of H_2S, but are not mediated via K^+_{ATP} channel. In our results, we can infer that H_2S gastroprotective effect is at least in part dependent on K_{ATP} activation.

Glibenclamide is an oral hypoglycemic agent that stimulates insulin secretion by blocking KATP channel producing hypoglycemia (Da Silva-Santos et al., 2002). The blood glucose level plays a critical role in the development of gastric lesions (Takeuchi et al., 1994). In this study, glibenclamide was administered in 2.5 ml/kg 0.02N NaOH containing 4% glucose to minimize hypoglycemia. We demonstrated that with this treatment glibenclamide did not change the

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glucose blood level when compared with saline (Gomes at al., 2006). Then, we can conclude that glibenclamide prevented the L-cysteine, H2S donors effects is not dependent on hypoglycemia.

Neuropeptide-containing afferent neurons are present in abundance in the gastric mucosa (Sternini et al., 1987). Activation of afferent neurons by a low dose of capsaicin exerts protection against a variety of ulcerogens (Holzer, 1998; or Ehrlich et al., 2004). Furthermore, high doses of capsaicin provoke neuron afferent denervation. In the context of the gastrointestinal tract, little is known about the effects of H_2S in the enteric nervous system. Earlier works have demonstrated that H_2S acts in enteric neurons. Schicho et al. (2006) found that guinea-pig and human enteric neurons expressed CSE and CBS, providing evidence that enteric neurons are able to synthesize H_2S. These authors also demonstrated that H_2S acts as a secretagogue in guinea-pig and human colon and involves activation of capsaicin sensitive nerve fibers and TRPV1 receptors. TRPV1 is an integrator of several painful stimuli and is thought to play a crucial role in gastrointestinal sensory and motor disorders (Gepetti and Trevisani, 2004). Our findings suggest that H_2S defensive effect on the gastric mucosa is dependent on capsaicin-sensitive sensory afferent fibers and TRPV1 receptors. Thus, desentization of afferent neurons with capsaicin and pretreatment with capsazepine abolished the effect of H_2S.

It is well-known that the activation of capsaicin-sensitive primary afferent neurons protects gastric mucosa for irritant injury, whereas ATP-sensitive potassium channel openers prevent the gastric damage induced by ethanol. However the interaction between capsaicin-sensitive primary afferent neurons and activation of KATP was not totally elucidated. Bratz et al. (2008) show that TRPV1 channels are functionally expressed in the vessels and mediate endothelium-dependent vasodilation through a mechanism involving nitric oxide and K^+ channels. Both ATP-sensitive and Ca^{2+}-activated K^+ channels have been implicated in mediating CGRP-induced relaxation for a variety of vascular beds. Glybenclamide antagonized CGRP

effects in mesenteric arteries (Nelson et al., 1990) and pulmonary vascular beds (Hood et al., 1991) suggesting the involvement of ATP-sensitive K^+ channels. Other possibility was that K_{ATP} and TRPV1 channels co-expressed on primary afferents neuron are involved in gastric protection. Chi et al. (2007) show that activation of K_{ATP} may play an important role in reversing the enhanced excitability produced by pro-inflammatory agents, such as PGE_2, in small to medium diameter capsaicin-sensitive sensory neurons. Rodrigues and Duarte (2000) suggest that the peripheral antinociceptive effect of morphine may result from activation of K_{ATP} channels, which may cause a hyperpolarization of peripheral terminals of primary afferents, leading to a decrease in action potential generation.

In summary, our results indicate that H_2S prevents ethanol-induced gastric damage. Although there are many mechanisms through which this effect can occur, our data supports the hypothesis that the activation of K_{ATP} channels and afferent neurons/TRPV1 receptors are of primary importance. These observations also raise the possibility of H_2S releasing agents being utilized to improve resistance to gastric mucosa injury.

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Footnotes section

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Legends for Figures

Figure 1. Hydrogen sulphide reduces ethanol-induced gastric damage. Mice were treated by gavage with L-cysteine alone or with propargylglycine (PAG) (panel *A*), NaHS (panel *B*) or Lawesson´s reagent (panel *C*) 30 min before ethanol 50% (0.5 ml $25g^{-1}$) administration. Control group was treated with saline only. The total area of macroscopic gastric lesions was determined after 1h. Results are expressed as the means ± s.e.m of at least five mice per group. *P<0.05; **P<0.01, when compared to the ethanol group; #P<0.01, when compared the L-cysteine 50 mg kg^{-1} group. ANOVA and Newman-Keuls test.

Figure 2. Photomicrographs of gastric mucosa (Magnifications, x100): (A) control (saline); (B) absolute ethanol, showing disruption of gastric gland superficial region with epithelial cell loss, intense hemorrhage; (C), ethanol 50% + NaHS (150 μmol kg^{-1}), (D) ethanol 50% + Lawesson´s reagent (27 μmol kg^{-1}), (E) ethanol 50% + L-cysteine (50 mg kg^{-1}), showing preservation of gastric mucosa. (F) Ethanol 50% + L-cysteine (50 mg kg^{-1}) + propargylglycine (50 mg kg^{-1}), showing diminished gastroprotective effect.

Figure 3. Reduction of ethanol-induced gastric damage by H_2S is mediated by K_{ATP} channels. Glibenclamide (3 and 10 mg kg^{-1}, i.p.) was injected 30 min before L-cysteine (50 mg kg^{-1}), H_2S donors (NaHS 150 μmol kg^{-1}; Lawesson´s 27 μmol kg^{-1}) or saline (sal) injections. Thirty minutes later, ethanol 50% (0.5 ml $25g^{-1}$) was administered. The control group was treated with saline alone. The total area of macroscopic gastric lesions was determined after 1h. Protective effect of L-cysteine (panel *A*), NaHS (panel *B*), and Lawesson´s reagent (panel *C*) was inhibited by glibenclamide. Results are expressed as the means ± s.e.mean of at least five mice per group.

*P<0.01, when compared to the ethanol group; #P<0.01, when compared the L-cysteine, or H_2S donors group + ethanol. ANOVA and Newman-Keuls test.

Figure 4. Reduction of ethanol-induced gastric damage by H_2S is mediated by sensory afferents fibers. Mice were treated with neurotoxic doses of capsaicin. After 8 days, desensitized animals were treated with vehicle (sal), L-cysteine (Cys, 50 mg kg^{-1}), NaHS (150 μmol kg^{-1}, p.o.) Lawessen's reagent (Law, 27 μmol kg^{-1}, p.o.). Thirty minutes later, ethanol 50% (0.5 ml $25g^{-1}$) was administered. The control group was treated with saline only. Macroscopic gastric damage total area was determined after 1h. The gastroprotective effect of L-cysteine, NaHS and Lawesson´s reagent was inhibited by ablation of sensory afferent fibers by capsaicin. Results are expressed as the means ± s.e.mean of at least eight mice per group. *P<0.05, when compared to the L- cysteine + ethanol group; ΨP<0.05, when compared to the Lawesson´s reagent + ethanol group; #P<0.05, when compared to the NaHS + ethanol group. ANOVA and Newman-Keuls test.

Figure 5. Reduction of ethanol-induced gastric damage by H_2S is mediated TRPV1 receptors. Capsazepine (10 mg kg^{-1}, i.p.) was injected 30 min before L-cysteine (50 mg kg^{-1}), H_2S donors (NaHS 150 μmol kg^{-1}; Lawesson´s 27 μmol kg^{-1}) or saline (sal) injections. Thirty minutes later, ethanol 50% (0.5 ml $25g^{-1}$) was administered. The control group was treated with saline only. Macroscopic gastric damage total area was determined after 1h. L-cysteine, NaHS and Lawesson´s reagent gastroprotective effect was inhibited by TRPV1 antagonist (capsazepine). Results are expressed as the means ± s.e.mean of at least eight mice per group. *P<0.05, when compared to the L- cysteine + ethanol group; ΨP<0.05, when compared to the Lawesson´s reagent + ethanol group; #P<0.05, when compared to the NaHS + ethanol group. ANOVA and Newman-Keuls test.

Table 1. Effect of H_2S donors (Lawesson´s reagent, NaHS.), or L-cysteine alone, or with propargylglycine (PAG) in ethanol-induced gastric microscopic damage.

Experimental group (N=6)	Hemorrhagic damage (score 0 - 4)	Oedema (score 0 - 4)	Epithelial cell loss (score 0 - 3)	Inflammatory cells (score 0 - 3)	Total (score 0-14)
Saline	0	0	0	0	0
Ethanol	3.5 (3 – 4)	4 (3 – 4)	3 (2 – 3)	0	0.5 (8 – 11)
Ethanol + Lawesson´s (27 μmol kg^{-1})	1 (0 – 1)*	1 (0 – 1)*	0 (0 – 1)*	0	2 (0 – 2)*
Ethanol + NaHS (150 μmol kg^{-1})	1 (0 – 2)*	1 (1 – 0)*	1 (1 – 2)*	0	2 (1 – 2)*
Ethanol + L-cysteine (50 mg kg^{-1})	1 (0 – 3)*	2 (1 – 2)	1 (1 – 3)	0	5 (2 – 7)*
Ethanol + L-cysteine + PAG (50 mg kg^{-1})	4 (3 – 4)$^{\#}$	4 (2 – 4)$^{\#}$	2 (2 – 3)	0	10 (8 – 11)$^{\#}$

Data shown are medians with minimum and maximal scores shown in brackets.

*P<0.05, vs ethanol group; $^{\#}P$<0.05, vs Ethanol + L-cysteine + Propargylglycine group. Kruskal-Wallis nonparametric test, followed by Dunn´s test was used for multiple comparisons for histological assessment.

Table 2- Effect of Lawesson´s reagent, NaHS, L-cyteine alone, or with propargylglycine (PAG) in ethanol (50%)-induced decrease in GSH and increase MDA concentration in gastric mucosa.

Experimental group	GSH (μg /g of tissue)	MDA (nmol / g of tissue)
Saline	490.7 ± 25.7	51.9 ± 3.4
Ethanol	227.5 ± 12.7 (*)	89.1 ± 3.2 (*)
Ethanol + Lawesson´s (27 μmol kg^{-1})	500.7 ± 33.6 (#)	56.3 ± 1.8 (#)
Ethanol + NaHS (150 μmol kg^{-1})	351.6 ± 29.0 (#)	48.6 ± 7.8 (#)
Ethanol + L-cysteine (50 mg kg^{-1})	484.3 ± 36.0 (#)	68.6 ± 8.6 (#)
Ethanol + L-cysteine (50 mg kg^{-1}) + PAG (50 mg kg^{-1})	288.1 ± 57.1 ($^{\Psi}$)	99.6 ± 5.3 ($^{\Psi}$)

(*) $p<0.05$, when compared with saline group. (#) $p<0.05$, when compared with Ethanol group. ($^{\Psi}$) $p<0.05$, when compared with Ethanol + L-cysteine group. ANOVA and Newman-Keuls test.

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Figure 1

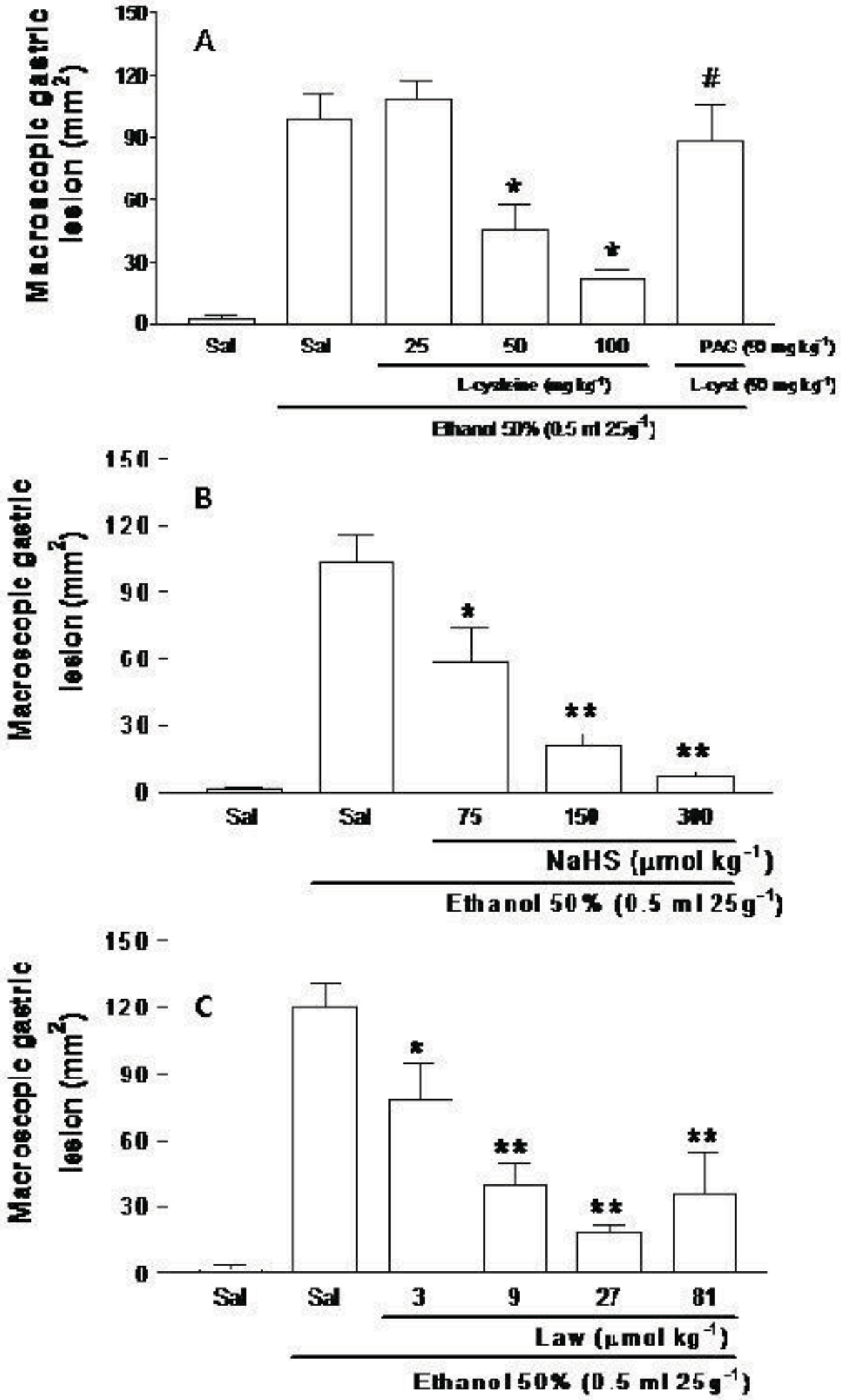

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Figure 2

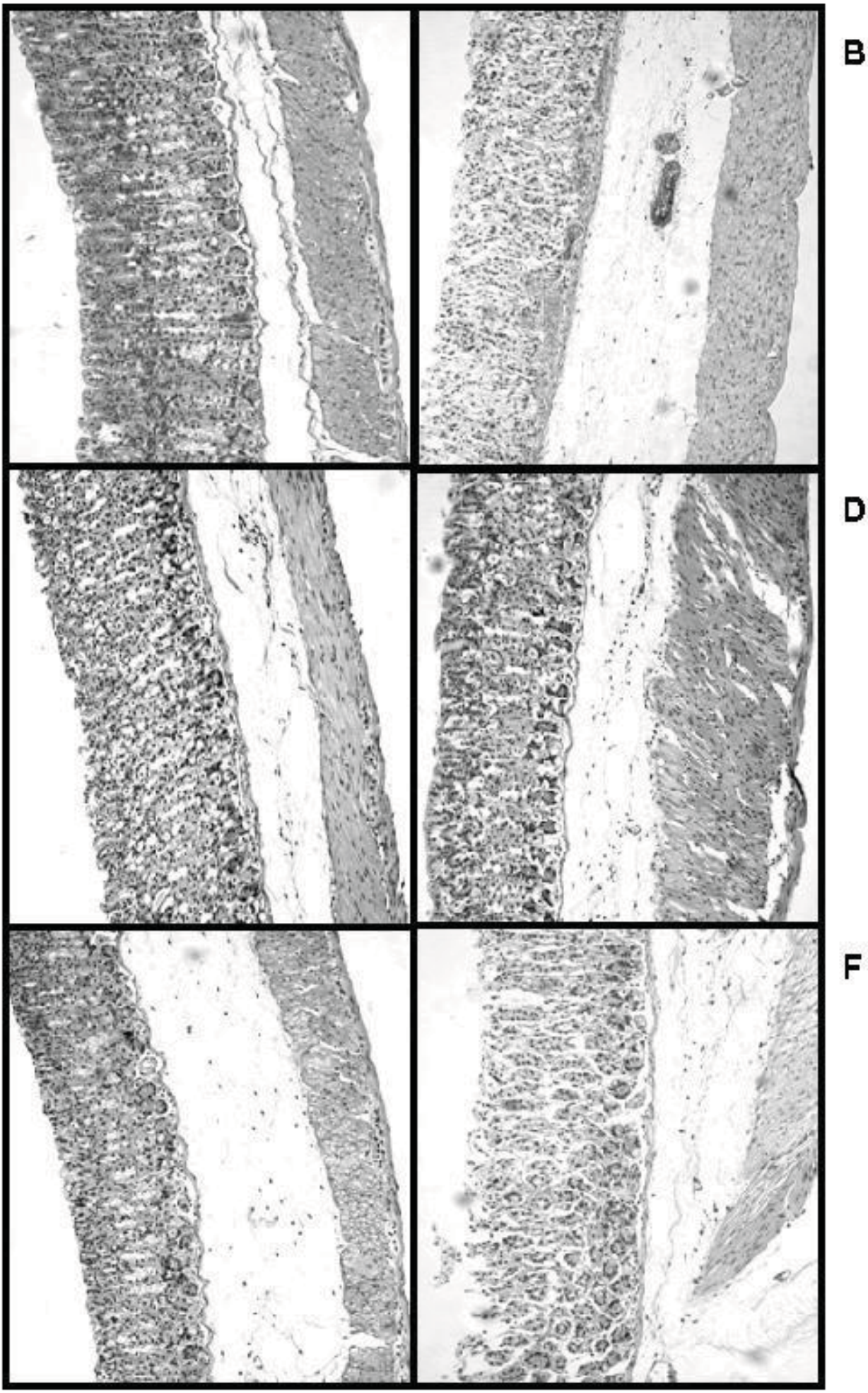

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Figure 3

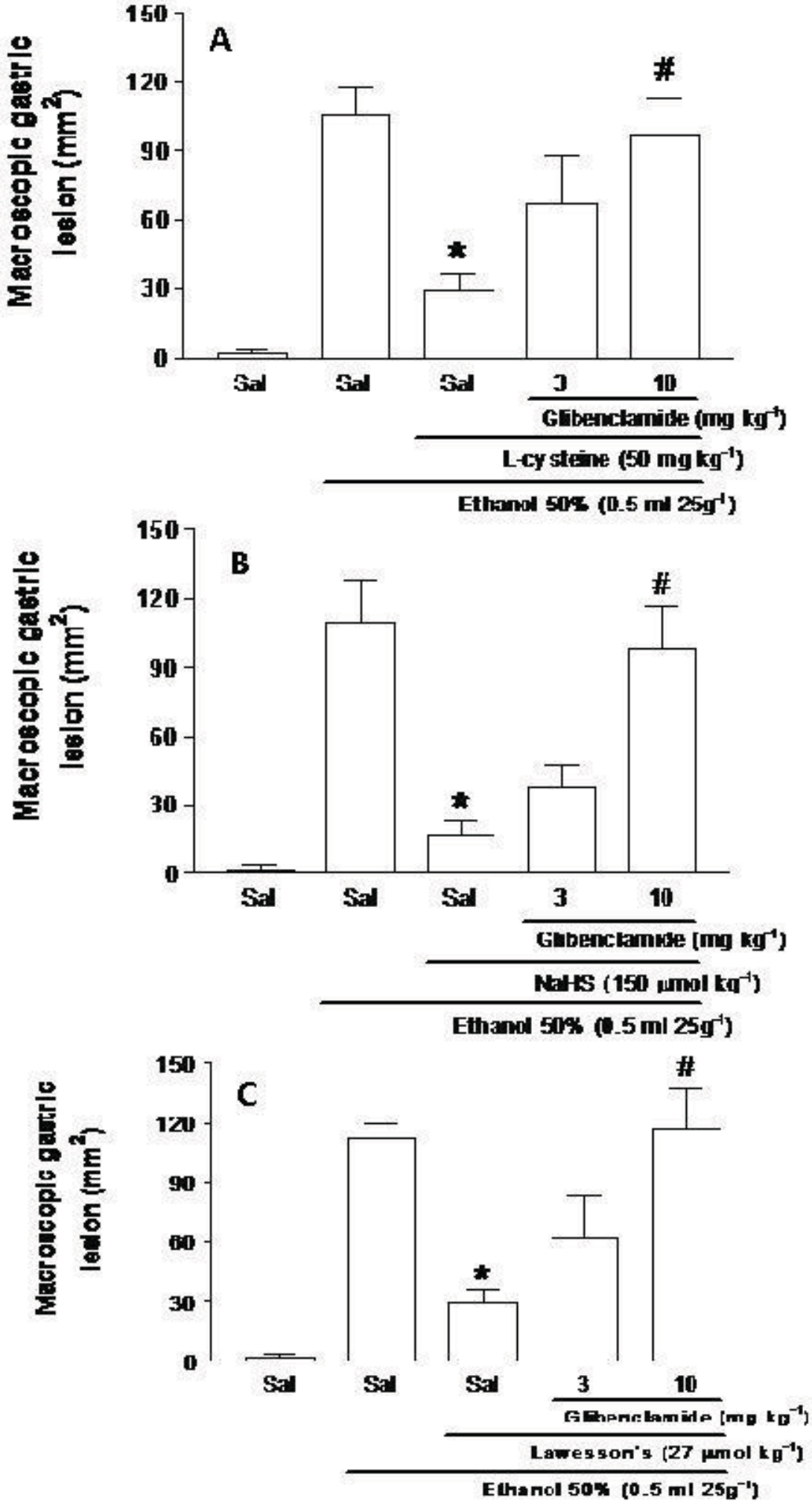

Figure 4

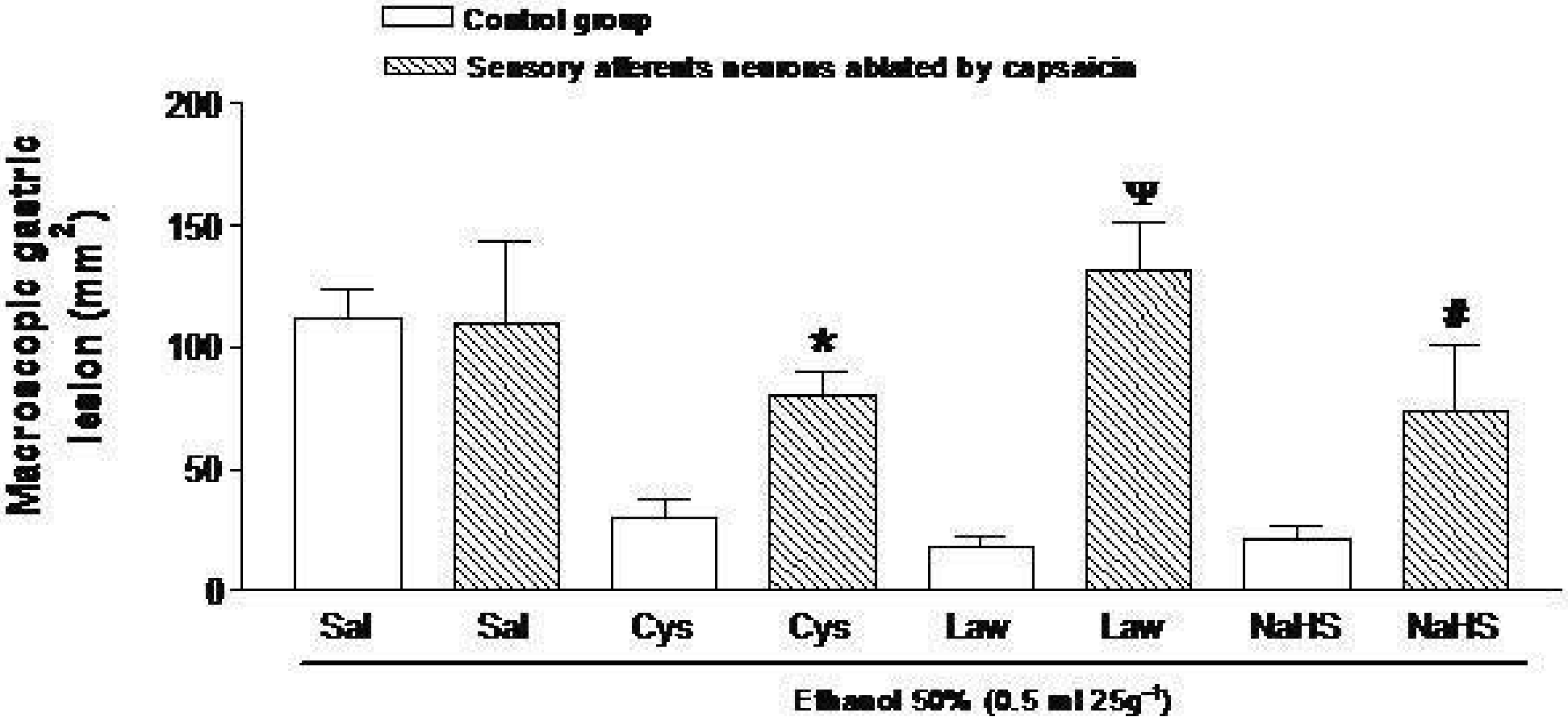

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Figure 5