Original article:

CITRIC ACID STRONGLY INHIBITS VISCERAL PAIN RESPONSE IN MICE

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ABSTRACT

Citric acid introduced into the stomach of mice at increasing concentrations of 0.1, 1 or 10 % (4.8 µM-0.48 mM; 95 µmol/kg–9.5 mmol/kg, 0.5 ml) caused dose-dependent inhibition of abdominal constrictions induced 1 h later by i. p. acetic acid injection by –51 % to -69.5 %. When administered at 10 % (0.48 mM, 0.5 ml) 15 min before nociceptive challenge, citric acid inhibited the nociceptive response by 96.8 %. Inhibition of the acetic acid-induced abdominal constrictions was also observed when lower doses of citric acid were introduced into the stomach (0.2 ml of 0.1-1 %; 38.1 µmol/kg-0.38 mmol/kg). The effect was evident as early as 5 min after administration of citric acid into the stomach and with the maximal effect being at 15-30 min after dosing. Lidocaine given orally 5 min prior to citric acid (1 %, 48 µM; 0.38 mmol/kg, 0.2 ml) prevented antinociception by citric acid, but lidocaine given 15 min before oral introduction of citric acid enhanced the citric acid-induced inhibition of the nociceptive response to acetic acid. The antinociceptive effect of orally administered citric acid (1 %, 48 µM; 0.38 mmol/kg, 0.2 ml) was increased by pre-treatment with propranolol (4 mg/kg, s. c.), yohimbine (4 mg/kg, s. c.), guanethidine (32 mg/kg, s. c.), but reduced after treatment with atropine (3 mg/kg, s. c.), which itself increased the nociceptive behavior. Similar inhibition of the acetic acid-induced nociceptive behavior was also observed when sodium citrate (pH 7.21) or 0.1 N HCl (pH 3) or 1 % sucrose solution (0.2 ml) was intragastrically given. It is suggested that citric acid might act to stimulate sensory afferents and that transmission of nociceptive information centrally leads to the activation of descending antinociceptive mechanism to a noxious stimulus.

Keywords: citric acid, intraperitoneal acetic acid, visceral nociceptive pain, mice

INTRODUCTION

Visceral abdominal pain is a common type of pain which is poorly understood. In contrast to somatic pain, the neurophysiologic mechanisms involved in visceral sensation are generally less well understood and the clinical management of visceral pain states is still limited (Joshi and Gebhart, 2000). Visceral pain results from activation of sensory afferent nerves innervating internal organs (Cervero and Laird, 1999). Viscerosensory axons are almost exclusively thinly myelinated A-delta and unmyelinated C fibers. The receptors exhibit chemosensitivity, thermosensitivity and/or mechanosensitivity. The gastrointestinal tract has rich sensory innervation comprising intrinsic sensory neurons contained entirely within the gastrointestinal wall, intestinofugal fibres that project to prevertebral ganglia and vagal and spinal afferents that project into the central nervous system. Afferent fibres convey sensory information from the upper gastrointestinal tract to the CNS via

vagal and splanchnic nerve pathways (Grundy, 2002). In the gastrointestinal tract, sensory nerves subserve protective functions. Capsaicin introduced into the rat stomach in very low concentrations in μM range which stimulated the peripheral endings of capsaicin sensitive sensory nerves effectively prevented gastric mucosal injury evoked by pylorus ligation, topical acidified aspirin or ethanol (Abdel-Salam et al., 1999; Szolcsányi and Barthó, 2001).

An intriguing question is whether a nociceptive stimulus applied to the gastric mucosa would affect visceral nociception evoked for example by i. p. injection of acetic acid in mice, a model of inflammatory visceral pain. In previous studies, capsaicin or piperine introduced into the stomach inhibited abdominal constrictions evoked by i. p. injection of acetic acid in mice. It would appear thus those nociceptive stimuli. In the present study we aimed to investigate whether citric acid would evoke a similar effect. Citric acid is a weak organic acid found in the greatest amounts in citrus fruits. It is a natural preservative and is also used to add an acidic (sour) taste to foods and soft drinks. Citric acid applied to the tip of the tongue in human subjects produced taste sensations and also irritation mediated via capsaicin-sensitive fibers since reductions in irritation and taste occurred following treatment with capsaicin (Gilmore and Green, 1993). Intraoral infusions of 0.1 M citric acid in awake, behaving rats elicited Fos-like immunoreactivity in the nucleus of the solitary tract (Travers, 2002). Citric acid 250 mM applied to the dorsal surface of the tongue in human caused irritation which involves acid-sensitive ion channels and vanilloid receptors (Dessirier et al., 2000).

The present study was therefore designed to test the effect of intragastric administration of citric acid on the visceral nociceptive response to intraperitoneal injection of dilute acetic acid in mice.

MATERIALS AND METHODS

Animals

Swiss male albino mice 22-25 g of body weight were used. Standard laboratory food and water were provided *ad libitum*. Experiments were performed between 9 am and 3 pm. The study was done in the Department of Pharmacology, National Research Centre, Cairo on February, 2006. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Equal groups of 6 mice each were used in all experiments.

Acetic acid-induced writhing

Separate groups of 6 mice each were administered vehicle (distilled water) or citric acid (0.01, 0.1, 1 or 10 %, 0.5 ml p. o.). After 1 h pretreatment interval, an i. p. injection of 0.6 % acetic acid was administered (Koster et al., 1959). The effect of citric acid (10 %, 0.5 ml, p. o., $n = 6$) administered 15 min before acetic acid was also studied. Other experiments were designed in an attempt to elucidate the dose and time-dependent effect of citric acid. The latter was given at 0.2 ml volume and 0.1 or 1 % concentration, 5 min, 15 min or 1 h prior to i. p. acetic acid injection ($n = 6$/group). Each mouse was then placed in an individual clear plastic observational chamber, and the total number of writhes made by each mouse was counted for 30 min after acetic acid administration.

Further experiments aimed to investigate the mechanisms by which citric acid exerts its anti-nociceptive effect. Citric acid at concentration of 1 % and 0.2 ml volume, p. o. was selected to be used in the subsequent experiments and administered 30 min prior to nociceptive challenge with i. p. acetic acid. Thus, the effect of the local anaesthetic lidocaine given 5 or 15 min prior to citric acid (1 %,

0.2 ml, p. o.) or vehicle was studied. Further, the effect of the beta adrenoceptor antagonist, propranolol (4 mg/kg, s. c.), the alpha-2 adrenoceptor antagonist yohimbine (5 mg/kg, s. c.), the adrenergic blocker, guanethidine (32 mg/kg, s. c.), the muscarinic acetylcholine receptor antagonist atropine (0.8, 1.6 or 3 mg/kg, s. c.) were examined on antinociception caused by citric acid. Antagonist drugs were administered 30 min before citric acid (1 %, 0.2 ml, p. o.) and i. p. administration of acetic acid was carried out 30 min after citric acid was given. In addition, effect of co-administered theophylline (10 or 30 mg/kg, s. c.) on the antinociceptive effect of orally administered citric acid (1 %, 0.2 ml, p. o.) was studied.

Moreover, we studied the effect of orally administered sodium citrate (pH 7.21; sodium hydroxide added to make pH 7.21 from pH 3.12) or 0.1 N HCl (pH 3) or 1 % sucrose solution (0.2 ml) on the abdominal constrictions caused by i. p. injection of acetic acid. Test solutions were given 30 min prior to nociceptive challenge with acetic acid.

Drugs and chemicals

Citric acid, atropine sulfate, yohimbine hydrochloride, propranolol hydrochloride and guanethidine hydrochloride (Sigma, St. Louis, USA) were used. Analytical-grade glacial acetic acid (Sigma, St. Louis, USA) was diluted with pyrogen-free saline to provide a 0.6 % solution for i. p. injection. All drugs were dissolved in isotonic (0.9 % NaCl) saline solution immediately before use. Stock solutions of capsaicin (10 mg/ml) contained 10 % ethanol, 10 % Tween 80, 80 % saline solution.

Statistical analysis

Data are expressed as mean ± S.E. Data were analyzed by one way analysis of variance, followed by a Tukey's multiple range test for *post hoc* comparison of group means. When there were only two groups a two-tailed Student's t test was used. For all tests, effects with a probability of $P < .05$ were considered to be significant.

RESULTS

Citric acid introduced into the stomach of mice at increasing concentrations of 0.1, 1 or 10 % (4.8 µM-0.48 mM; 95 µmol/kg–9.5 mmol/kg, 0.5 ml) caused a dose-dependent inhibition of abdominal constrictions induced 1 h later by i. p. acetic acid injection by −51 % to -69.5 %. The lower concentration of 0.01 % of acetic acid was without effect on the nociceptive response. Marked inhibition of the nociceptive response by 96.8 % was observed when citric acid at 10 % (0.48 mM, 0.5 ml) was orally introduced 15 min before nociceptive challenge (Fig. 1).
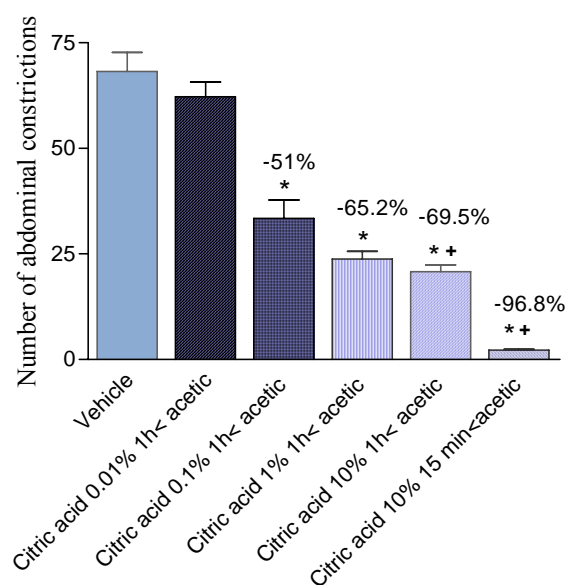

Figure 1: Inhibition of the acetic acid-induced abdominal constrictions in mice by citric acid administered orally. Citric acid was introduced into the stomach at concentrations of 0.01-10 % (0.48 µM-0.48 mM; 9.5 µmol/kg–9.5 mmol/kg, 0.5 ml) 1 h prior to testing. The effect of citric acid (10 % solution, 0.5 ml) administered 15 min prior to acetic acid challenge is also shown (last column). Data expressed as mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 vs. vehicle
+ : p<0.05 vs. 0.1 % citric acid.

Lower doses of citric acid were also effective in inhibiting the visceral nociceptive response to i. p. acetic acid. Thus citric acid introduced into the stomach at 0.2 ml of 0.1-1 % solution (4.8 µM-48 µM; 38.1 µmol/kg-0.38 mmol/kg), 5, 15 or 60 min prior to nociceptive challenge reduced the number of abdominal constrictions by 9.4-19.5 %, 30.3–46.3 % and by 14.3-20.5 %, respectively (Fig. 2). It would thus appear that the analgesic effect of citric acid is both dose and time dependent, with the effect being evident as early as 5 min after administration of citric acid into the stomach and with the maximal effect being at 15-30 min after dosing. Accordingly in further experiments, citric acid was used in a concentration of 1 % and at 30 min prior to nociceptive testing.
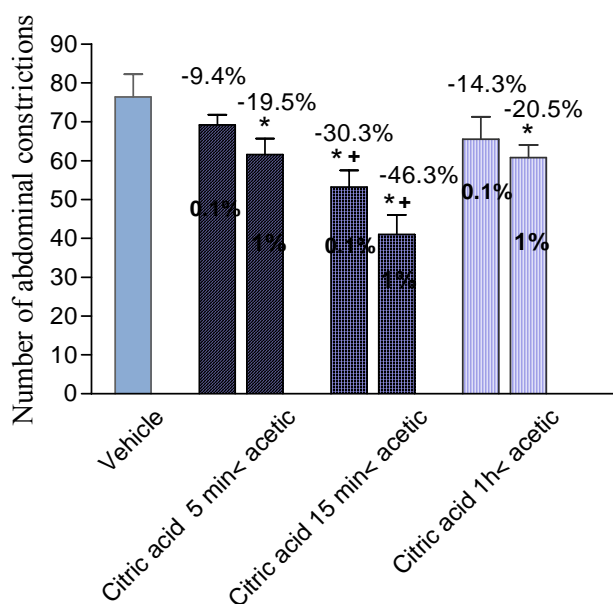
Lidocaine (2 %, 0.1 ml) itself given orally 5 min prior to intragastric saline administration (0.2 ml) reduced the number of abdominal constrictions caused 30 min later by i. p. acetic acid by 44.1 %. When lidocaine was given 5 min prior to intragastric citric acid (1 %, 48 µM; 0.38 mmol/kg, 0.2 ml) no further inhibition of the nociceptive reaction was noted. Meanwhile, lidocaine given 15 min before oral introduction of citric acid enhanced the citric acid-induced inhibition of the nociceptive response to acetic acid (Fig. 3).
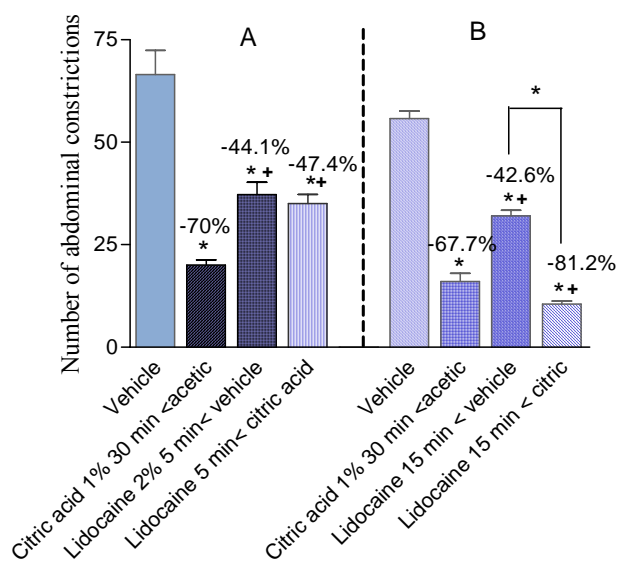

Figure 3: Effect of lidocaine (2 %, 0.1 ml, p. o.) on antinociception induced by orally administered citric acid (1 %, 48 µM; 0.38 mmol/kg, 0.2 ml) in the abdominal constriction assay in mice. Lidocaine was administered 5 (A) or 15 min (B) prior to citric acid. The nociceptive challenge with i. p. acetic acid was carried out 30 min after citric acid administration.
* : p<0.05 vs. vehicle and between different groups as shown in figure
+ : p<0.05 vs. citric acid group.

Figure 2: Effect of citric acid introduced into the stomach at 0.2 ml and concentrations of 0.1 or 1 % solution (4.8 µM or 48 µM; 38.1 µmol/kg or 0.38 mmol/kg) on the acetic acid-induced abdominal constrictions in mice. Citric acid was given 5, 15 or 60 min prior to nociceptive challenge. Data expressed as mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 vs. vehicle
+ : p<0.05 vs. corresponding concentration of citric acid at 5 min or 1 h.

Atropine administered at 0.8 or 1.6 mg/kg, s. c., had no effect on the analgesic action of citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) (Fig. 4).
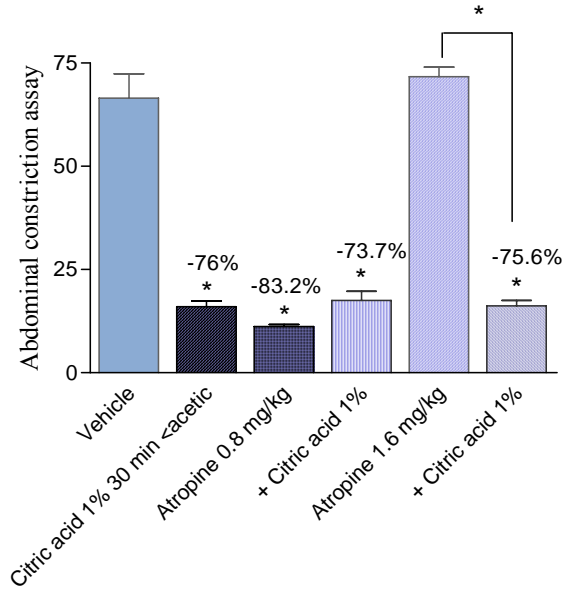
At a higher dose of 3 mg/kg, atropine itself enhanced visceral pain and masked the antinociceptive effect of citric acid (Fig. 5).

Figure 4: Effect of atropine (0.8 or 1.6 mg/kg, s. c.) on antinociception induced by orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) in the abdominal constriction assay in mice. Atropine was administered 30 min before citric acid (1 %, 0.2 ml, p. o.) and i. p. administration of acetic acid was carried out 30 min after citric acid was given. Data represent mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 compared to vehicle and between different groups as shown in the figure
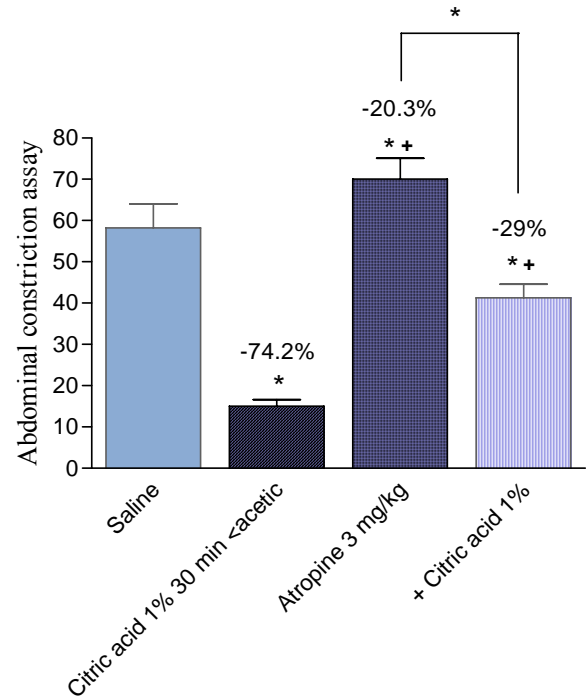+ : p<0.05 vs. citric acid group.

Figure 5: Effect of atropine (3 mg/kg, s. c.) on antinociception induced by orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) in the abdominal constriction assay in mice. Atropine was administered 30 min before citric acid (1 %, 0.2 ml, p. o.) and i. p. administration of acetic acid was carried out 30 min after citric acid was given. Data represent mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 compared to vehicle and between different groups as shown in the figure.

The antinociceptive effect of orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) was increased by pre-treatment with propranolol (4 mg/kg, s. c.), yohimbine (4 mg/kg, s. c.), guanethidine (32 mg/kg, s. c.) (Fig. 6).
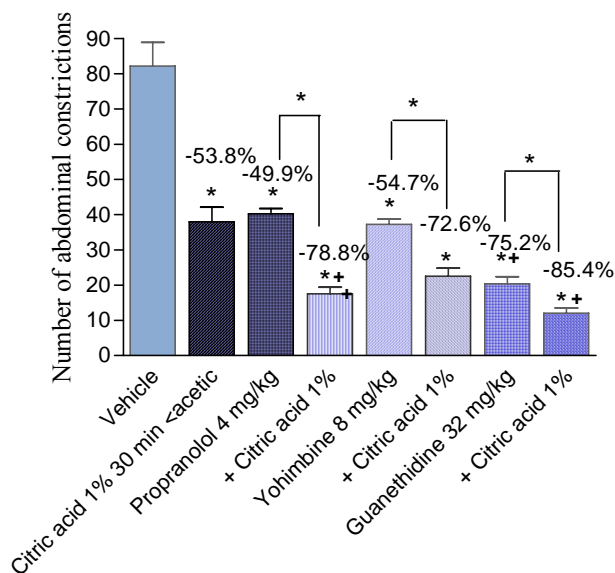
The antinociceptive effect of orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) was reduced by pre-treatment with theophylline at 30 mg/kg (Fig. 7).
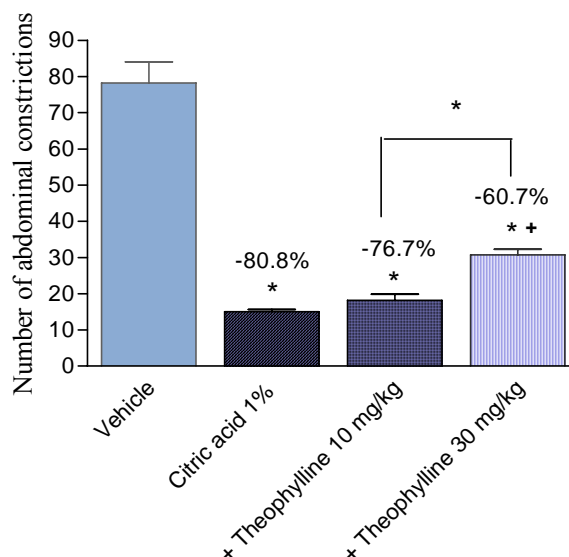
Figure 6: Effect of propranolol (4 mg/kg, s. c.), yohimbine (4 mg/kg, s. c.) or guanethidine (32 mg/kg, s. c.) on antinociception caused by orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) in the abdominal constriction assay in mice. Antagonist drugs were administered 30 min before citric acid (1 %, 0.2 ml, p. o.) and i. p. administration of acetic acid was carried out 30 min after citric acid was given. Data represent mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 compared to vehicle and between different groups as shown in the figure
+ : p<0.05 compared to citric acid group.

Figure 7: Effect of theophylline (10 or 30 mg/kg, s. c.) on antinociception caused by orally administered citric acid (1 %, 48 μM; 0.38 mmol/kg, 0.2 ml) in the abdominal constriction assay in mice. Theophylline was co-administered with citric acid 30 min before i. p. injection of acetic acid. Data represent mean ± S.E and percent inhibition (%) compared to the vehicle-treated group.
* : p<0.05 compared to vehicle and between different groups as shown in the figure
+ : p<0.05 compared to citric acid group.

Inhibition of the acetic acid-induced nociceptive behavior was also observed when sodium citrate (0.2 ml, pH 7.21) or 0.1 N HCl (0.2 ml, pH 3) (Fig. 8) or 1 % sucrose solution (0.2 ml) was intragastrically given (Fig. 9). There was an additive antinociception following the administration of citric acid and sucrose solution (Fig. 9).
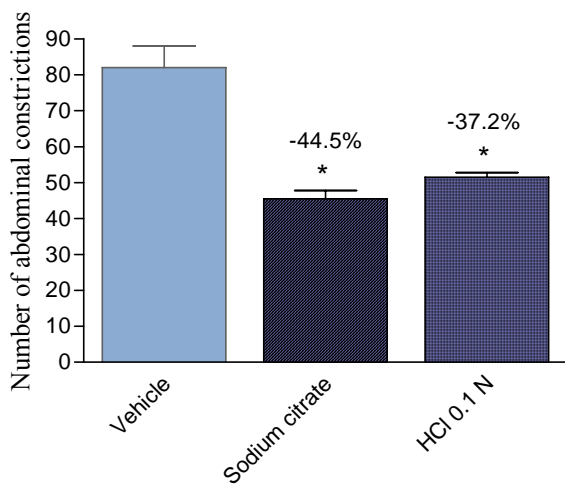
Figure 8: Effect of orally administered sodium citrate (pH 7.21) or 0.1 N HCl (pH 3) on the abdominal constrictions caused by i. p. injection of acetic acid. Test solutions were given 30 min prior to nociceptive challenge with acetic acid.
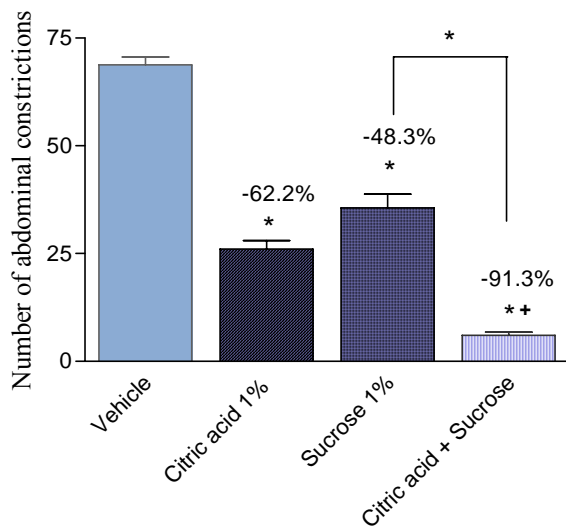* : $p < 0.05$ compared to vehicle.

Figure 9: Effect of orally administered 1 % sucrose solution (0.2 ml) on the abdominal constrictions caused by i. p. injection of acetic acid and on the antinociception caused by orally administered citric acid (1 %, 48 µM; 0.38 mmol/kg, 0.2 ml). Test solutions were given 30 min prior to nociceptive challenge with acetic acid.
* : $p < 0.05$ compared to vehicle and between different groups as shown in the figure
+ : $p < 0.05$ compared to citric acid group.

DISCUSSION

The present study indicated for the first time that the nociceptive behavioral response to noxious peritoneal stimulus (acetic acid) decreased following intragastric administration of citric acid in mice. Inhibition of the acetic acid-induced nociceptive behavior was also observed when sodium citrate (pH 7.21) or 0.1 N HCl (pH 3) or 1 % sucrose solution was intragastrically given. It would thus appear that a number of noxious and gustatory stimuli interfere with processing of noxious visceral stimulation at a distant site in the gastrointestinal tract. Gustatory and other oral sensory signals appear to trigger neural reflexes. In human subjects, oral application of sucrose 0.07, 0.28, 1.12 M or citric acid solutions 0.002, 0.008, 0.032 M elicited an increase in heart rate within 5 sec that peaked 40 sec and declined in 801-100 sec after application (Horio, 2000). In the present study, however, the antinociceptive effect of citric acid was not due to sympathetic or cholinergic reflexes. In previous studies, intragastric administration of capsaicin or piperine inhibited abdominal constrictions evoked by i. p. acetic acid in mice (Abdel-Salam, 2006, Abdel-Salam et al., 2007).

It has been shown that various nociceptive intestinal stimuli, e. g. chemical peritoneal stimulation by hydrochloric acid, mechanical stimulation of the small and large intestines or direct electrical stimulation of mesenteric or splanchnic afferents suppress gastric motility via a spino-vagal reflex mechanism composed of spinal afferents in the sympathetic nerves, spinobulbar ascending pathways, and vagal nonadrenergic inhibitory fibers to the stomach (Glise and Abrahamsson, 1980). It has been suggested that peripheral sensory input, such as noxious stimulation, might be a mechanism by which descending inhibitory system is physiologically activated (Gear et al., 1999). Studies also indicated that visceral noxious stimuli can result in inhibition of somatic inflammatory pain, e. g. an in-

traperitoneal acetic acid produced long-lasting inhibition of formalin-evoked somatic inflammatory pain behavior in mice. This effect was naloxone insensitive, but blocked by the 5-hydroxytryptamine type 2A/2C receptor antagonists (Kurihara et al., 2003).

Sensations from the stomach are conveyed to the central nervous system by capsaicin-sensitive fibers. Sensory neurons signal chemical noxae to the brain, a task that is not confined to spinal afferents because vagal afferents communicate gastric acid and peripheral immune challenges to the brainstem and in this way elicit autonomic, endocrine, affective and behavioral reactions (Holzer, 2004a, b). In this context, noxious gastric distention induced the expression in the nucleus of the solitary tract of c-fos, a marker for activity following noxious somatic or visceral stimulation (Traub et al., 1996). Increased c-fos transcription in the nucleus of the solitary tract and the spinal cord also followed intragastric capsaicin (3.2 mM; 2 ml) administration. The nociceptive information being processed both by gastric vagal and intestinal spinal afferents (Holzer et al., 2005). Vagal afferent input from the acid-threatened gastric mucosa leads to the activation of subcortical brain nuclei that are involved in emotional, behavioral, neuroendocrine, autonomic and antinociceptive reactions to a noxious stimulus (Michl et al., 2001). There is also evidence of a descending modulation of spinal visceral nociceptive transmission, whereby electrical and/or chemical (glutamate) stimulation of periaqueductal gray or rostral ventromedial medulla (Giesler and Liebeskind, 1976; Ness and Gebhart, 1987; Zhuo et al., 2002) or thalamic mucleus submedius (Yang and Follett, 2003) attenuated the neuronal responses to a noxious visceral stimulus (colorectal distension).

Citric acid has sensory properties. In human subjects, the application of citric acid to the tongue elicited taste sensations and irritations mediated via capsaicin-sensitive fibers since reductions in irrita-tion and taste occurred following treatment with capsaicin (Gilmore and Green, 1993). Citric acid 250 mM applied to the dorsal surface of the tongue in human caused irritation which involves acid-sensitive ion channels and vanilloid receptor mechanism (Dessirier et al., 2000). Intraoral infusions of 0.1 M citric acid in awake, behaving rats elicited Fos-like immunoreactivity in regions of the nucleus of the solitary tract in that receive input from orosensory afferents and also in a location that mainly receives primary afferent input from the vagus nerve. These results suggest that strong gustatory stimuli can influence visceral afferent systems (Travers, 2002).

On the other hand, glucose or sucrose solutions administered orally provide effective analgesia for procedural pain in neonates (Stevens et al., 2004; Rogers et al., 2006). This analgesia with sugar solutions can be decreased by opioid or 5HT2A serotonergic receptor antagonists (Reboucas et al., 2005). It was also suggested that glucose does not directly interact with Mu opioid receptors in an *in vitro* expression system and that the purported interaction between glucose and the opioid system may be an indirect one, involving release of endogenous opioids (Kracke et al., 2005). Intra-oral sucrose activates neurons in the periaqueductal gray and nucleus raphe magnus, two key brainstem sites critically involved in descending pain modulation (Anseloni et al., 2005). The analgesic effect of sucrose intake depends on the concentration of sucrose solution and on the time during which the solution is consumed (Segato et al., 2005).

Although in the present study, that citric acid and other test solutions were administered into the stomach through orogastric tube, it is also possible that the observed phenomenon represents gustatory activation of the afferent limb of visceral reflex circuits. Projections from somatosensory neurons throughout the oral cavity, and from visceral neurons in the gut, intermingle with gustatory neurons in the

NST (Whitehead and Frank, 1983) and GC (Barnett et al., 1995), suggesting that interactions should occur between taste and other systems. It is also possible that the observed antinociceptive response to intragastric stimuli reflects a systemic response to the release into the circulation of anti-inflammatory peptide. In this context, evidence has been provided that beyond evoking a local inflammatory reaction, antidromic electrical or orthodromic chemical stimulation of capsaicin-sensitive sensory nerve fibers dorsal roots, or sciatic nerve elicits a systemic anti-inflammatory effect as well. The development of this unorthodox systemic humoral response was found to be due to somatostatin release from sensory nerve terminals (Thán et al., 2000).

In conclusion, the present study provides the first evidence that the nociceptive behavioral response to noxious peritoneal stimulation with acetic acid in mice could be modulated following intragastric administration of citric acid. It is suggested that the antinociceptive effects of citric acid reflects the activation of a descending inhibitory pain pathway in response to visceral noxious stimulation and transmission of visceral nociceptive information centrally.

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