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HEPATOPROTECTIVE EFFECTS OF CITRIC ACID AND ASPARTAME ON CARBON TETRACHLORIDE-INDUCED HEPATIC DAMAGE IN RATS

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ABSTRACT

The aim of this study was to investigate the effect of citric acid or the sweetening agent aspartame on the CCl_4-induced hepatic injury in rats. Citric acid (10 mg/kg, 100 mg/kg or 1000 mg/kg), aspartame (0.625 or 1.25 mg/kg) or silymarin (25 mg/kg) was given once daily orally simultaneously with CCl_4 and for one week thereafter. The administration of citric acid at 100 mg/kg or 1000 mg/kg to CCl_4-treated rats reduced elevated plasma ALT by 44.1-63.3 %, AST by 47.8-70.6 %, ALP by 41.7-67.2 %, respectively compared to controls. Aspartame at 0.625 or 1.25 mg/kg reduced plasma ALT by 39.8-52.0 %, AST by 43.2-52.4 % and ALP by 50.0-68.5 %, respectively. Meanwhile, silymarin at 25 mg/kg reduced ALT, AST and ALP levels by 52.7, 62.2 and 64.7 %, respectively. On histology, citric acid at 1000 mg/kg resulted in near normalization of liver tissue. Vacuolar degeneration and necrosis were markedly reduced by 1.25 mg/kg aspartame. These results indicate that treatment with citric acid or the sweetening agent aspartame protects against hepatocellular necrosis induced by CCl_4.

Keywords: citric acid, aspartame, silymarin, acute hepatic injury, carbon tetrachloride, rat

INTRODUCTION

Citric acid, 2-hydroxy-1,2,3-propanetricarboxylic acid, is a weak organic acid found in the greatest amounts in citrus fruits such as lemon, grapefruit, tangerine and orange. It is an intermediary substance in oxidative metabolism, being a component of the tricarboxylic acid cycle. Citric acid is used as a natural preservative and also to add an acidic (sour) taste to foods and soft drinks (Grigor et al., 2002). Citric acid possesses sensory properties that is, when applied to the tip of the tongue in human subjects, citric acid produced taste sensations and also irritation which are mediated *via* capsaicin-sensitive fibers since reductions in irritation and taste occurred following treatment with capsaicin (Gilmore and Green, 1993). Citric acid applied to the dorsal surface of the tongue in human at the concentration of 250 mM caused irritation which involves acid-sensitive ion channels and vanilloid receptors (Dessirier et al., 2000). In awake, behaving rats, intraoral infusions of 0.1 M citric acid elicited Fos-like immunoreactivity in the nucleus of the solitary tract that receives 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). It has been shown that gustatory and other oral sensory signals can trigger neural reflexes (Horio, 2000). In a previous study, citric acid introduced into the stomach of mice at increasing concentrations of 4.8 μM-0.48 mM, resulted in inhibition of the nociceptive behavior induced by subse-

quent acetic acid injection into the peritoneal cavity. This phenomenon is also seen when sucrose solution was intragastrically given, which suggested that a number of noxious and gustatory stimuli interfere with processing of noxious visceral stimulation at a distant site in the gastrointestinal tract (Abdel-Salam and Baiuomy, 2008). The dipeptide aspartame (N-L-alpha-aspartyl-L-phenylalanine, 1-methyl ester; alpha-APM) is a widely consumed artificial sweetener. Sweet taste information results in c-Fos expression in the nucleus of the solitary tract (Streefland et al., 1996) and central transmission of sweetness information elicits insulin release (Tonosaki et al., 2007). In light of the above the present study was designed to test whether oral administration of citric acid or the low-calorie sweetener aspartame would modulate the hepatic damage produced by the hepatotoxin CCl_4 in rats.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats of either sex, weighing 120 g (age: 10 weeks) were used throughout the experiments and fed with standard laboratory chow and water *ad libitum*. 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).

Drugs and chemicals

Carbon tertrachloride (BDH Chemicals, England), citric acid, aspartame (Sigma, St. Louis, USA) and silymarin (SEDICO, ARE), were used in the experiments. The doses for rats used were equivalent to the human dose according to Paget and Barnes (1964) conversion tables.

The carbon tetrachloride model of hepatic damage

The rats were divided into 8 equal groups, 6 rats each. Groups 1-7, received CCl_4 in olive oil (1:1, vol/vol) at a dose of 2.8 ml/kg through orogastric tube. Starting on the first day of CCl_4 administration, rats were treated with citric acid (10 mg/kg, 100mg/kg, 1000 mg/kg, 1.25 ml, p.o.), aspartame (0.625 or 1.25 mg/kg, 0.25 ml, p.o.), silymarin (25 mg/kg) or saline once daily orally and for 7 days thereafter. All treated rats were administered half the initial dose of CCl_4, 3 days after the first administration of CCl_4 so as to maintain hepatic damage. In addition, an 8^{th} group of rats (n = 6) received the vehicle (olive oil) at 2.8 ml/kg followed 3 days later by an additional dose of 1.4 ml/kg olive oil. Rats had free access to food and drinking water during the study. After 7 days of CCl_4 or olive oil administration, rats were killed by cervical dislocation after being anaesthetized with ether.

Biochemical assessment

At the end of the experiments, blood samples were obtained from the retro-orbital vein plexus, under ether anaesthesia. ALT and AST activities in serum were measured according to Reitman-Frankel colorimetric transaminase procedure (Crowley, 1967), whereas colorimetric determination of ALP activity was done according to the method of Belfield and Goldberg (1971), using commercially available kits (BioMérieux, France).

Histological and histochemical studies

Livers of all animals were dissected immediately after death. The specimens were then fixed in 10 % neutral-buffered formalin saline for 72 hours at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Serial sections of 6 µm thick were cut and stained with haematoxylin and eosin for histopathological investigation. Images were captured and

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processed using Adobe Photoshop version 8.0.

Statistical analysis

All results are expressed as mean ± SE. Comparison of the values before and after CCl_4 was made by paired Student's t-test. Multiple group comparisons were performed by ANOVA followed by Duncan test. $P < 0.05$ was considered statistically significant.

RESULTS

Biochemical changes

In rats treated with CCl_4, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in plasma were markedly raised by 227.1 % (181.7 ± 13.6 *vs* 80.3 ± 6.8 U/l), 265.7 % (188.4 ± 11.0 *vs* 71.0 ± 4.0 U/l) and 289.2 % (147.5 ± 10.1 *vs* 51.9 ± 2.8 U/l), indicating the severity of hepatic injury caused by CCl_4. Citric acid given at time of CCl_4 administration at doses of 10 mg/kg did not significantly alter the plasma enzyme levels. The administration of citric acid at 100 mg/kg or 1000 mg/kg to CCl_4-treated rats reduced the elevated plasma ALT by 44.1-63.3 %, AST by 47.8-70.6 %, ALP by 41.7-67.2 %, respectively compared to controls. Aspartame at 0.625 or 1.25 mg/kg reduced plasma ALT by 39.8-52.0 %, AST by 43.2-52.4 %, ALP by 50.0-68.5 %, respectively. Silymarin treatment at 25 mg/kg reduced the levels of ALT, AST and ALP by 52.7, 62.2 and 64.7 %, respectively (Table 1).

Histological findings

The liver of control rats revealed the normal characteristic architecture (Fig. 1A, B). The liver tissue of rats treated with CCl_4 only, showed congestion of central veins, dilatation and congestion of portal vein and fibrosis (Fig. 1C, D). The liver tissue from rats treated with citric acid (10 mg/kg) and CCl_4, still exhibited dilatation and congestion of both portal and central vein as well as the fibrosis (Fig. 2A, B). Examination of liver sections from rats given citric acid in a dose of 100 mg/kg revealed normal size and shape of the central vein and there was marked reduction of dilatation and congestion of the portal vein. Figure 2 E & F show the effect of citric acid administered at the dose of 1000 mg/kg, where normalization of liver tissue is observed.

Table 1: Effect of citric acid or aspartame on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in CCl_4-treated rats

	Vehicle	CCl_4 (control)	CCl_4 + citric acid 10 mg/kg	CCl_4 + citric acid 100 mg/kg	CCl_4 + citric acid 1000 mg/kg	CCl_4 + aspartame 0.625 mg/kg	CCl_4 + aspartame 1.25 mg/kg	CCl_4 + silymarin 25 mg/kg
ALT (U/l)	71.0 ± 4	188.4 ± 11.0	159.3 ± 8.6	98.4 ± 6.1$^{*+\#}$	55.5 ± 4.9^{*+}	107.1 ± 6.9$^{*+\#}$	89.7 ± 5.6^{*+}	71.2 ± 5.4^{*+}
AST (U/l)	80.3 ± 6.3	181.7 ± 13.6	169.0 ± 12.6	101.6 ± 7.6^{*+}	66.8 ± 3.8^{*+}	109.3 ± 7.6$^{*+\#}$	87.2 ± 6.2^{*+}	86.0 ± 4.5^{*+}
ALP (U/l)	51.9 ± 2.8	147.5 ± 10.1	133.9 ± 10.8	86.9 ± 5.6$^{*+\#}$	48.6 ± 4.7^{*+}	74.0 ± 6.0$^{*+\#}$	46.3 ± 4.3^{*+}	51.9 ± 5.0^{*+}

*: $p<0.05$ vs CCl_4 control group; +: $p<0.05$ vs CCl_4 + citric acid 10 mg/kg-treated group; #:$p<0.05$ vs silymarin-treated group or *vs* CCl_4 + citric acid 1000 mg/kg-treated group

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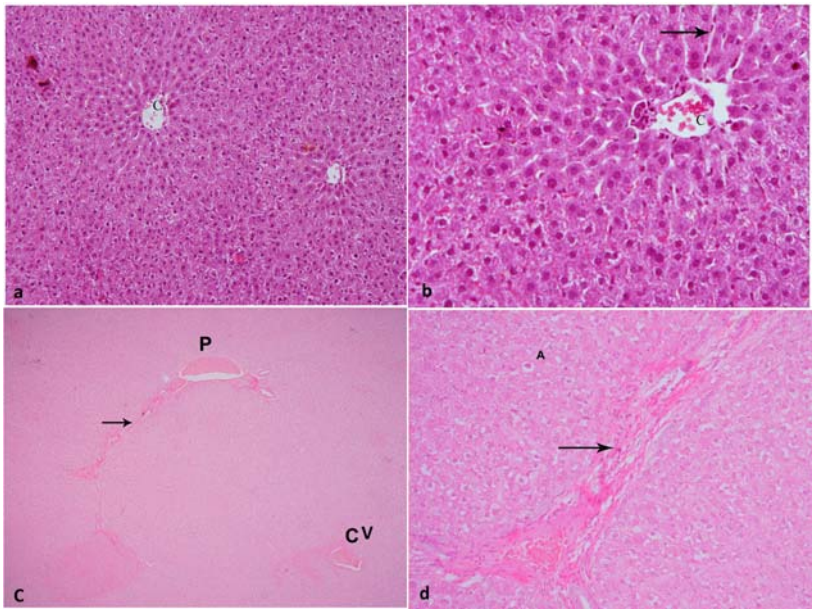

Figure 1 (a, b): A photomicrograph of a section of liver tissue showing the normal structure of liver tissue, where the cells are arranged in cords radiating from the central vein (c) forming the classic lobule without sharp demarcation between adjacent lobules; and the cords of hepacytes that are of thickness of 1-2 cells and separated from each other by equal-sized blood sinusoids. The lumens of these sinusoids contain blood cells and nuclei of kupffer cells (arrow). **(c):** A photomicrograph of a section of liver tissue of a control CCl_4-treated rat, showing congestion of central veins (CV), dilatation and congestion of portal vein (P) and fibrosis in the form of a septum extending from the portal area in the parenchyma of liver (arrow). The fibrous septum is composed mainly of collagenous fibers and fibrocytes (arrow). Some of the hepatocytes beside the septum show apoptosis (A). The normal architecture of the liver tissue is markedly disturbed **(d)**.

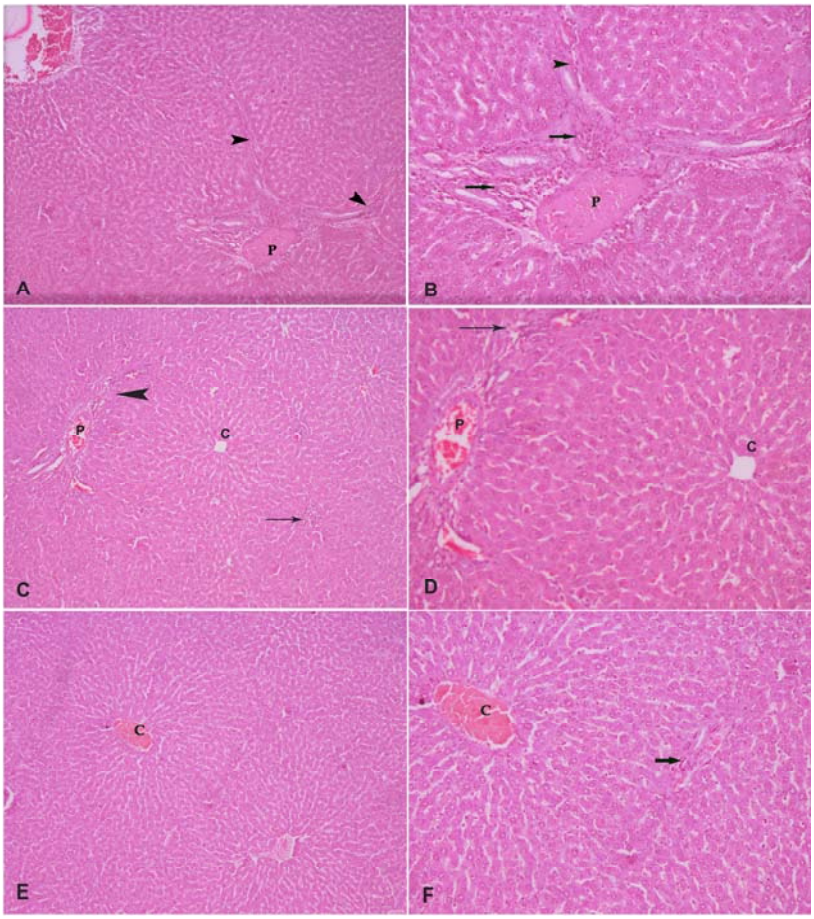

Figure 2: (A) A photomicrograph of a section of liver tissue from a rat treated with CCl_4 and citric acid (10 mg/kg), showing that dilatation and congestion of both portal and central vein are still present as well as the fibrosis especially at the portal area extending from it outwards (arrow head). In section **(B)** cellular infiltrates (arrow) are seen at the area of fibrosis, which is composed mainly of collagen fibers (arrow head). Section **(C)** shows the effect of citric acid at a dose of (100 mg/kg) where the central vein regains its normal size and shape (c) and there is marked reduction of dilatation and congestion of portal vein (P). However, there is still fibrous tissue extending from the portal area (arrow head) and at the boundary of the classic lobule (arrow). Section **(D)** is a higher magnification of the previous section showing that the fibrous tissue is made up of collagenous fibers and minimal cellular infiltrate (arrow). Sections **(E & F)** show the effect of citric acid at the dose of 1000 mg/kg, where normalization of liver tissue is observed, although there are remnants of fibrous tissue at the boundary of the lobules (arrow).

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Figure 3A & B show a section of liver tissue treated with aspartame at the dose of 0.625 mg/kg and CCl_4 showing necrosis, vacuolar degeneration and fibrosis. With the higher dose of 1.25 mg/kg of aspartame, hepatocyte degeneration and necrosis were markedly reduced (Fig. 3C & D).

Discussion

The present study provided the first evidence that both the natural preservative and food additive citric acid and the artificial sweetener aspartame were able to lessen the degree of hepatocellular damage caused by CCl_4 in rats. The release of hepatocelluar enzymes into blood was decreased and histological indices of hepatocelluar injury were reduced by either agent in a dose-dependent manner.

Citric acid is used as a natural preservative and also to add an acidic (sour) taste to foods and soft drinks (Grigor et al., 2002). Citric acid possesses sensory properties. In the airways, citric acid excites C-fibres and mimic many of the effects of capsaicin, including pain, bronchoconstriction, cough, and sensory neuropeptide release (Fox et al., 1993, 1995). In the oral cavity, the taste sensation caused by citric acid is mediated *via* capsaicin-sensitive fibers (Gilmore and Green, 1993; Geppetti et al., 1993; Dessirier et al., 2000) and the responses of gustatory neurons in the nucleus tractus solitarius to citric acid (0.03 M), decreased after lingual application of capsaicin (Simons et al., 2003). 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 central nervous system *via* vagal and splanchnic nerve pathways (Grundy, 2002; Holzer et al., 2005). In the meantime, local efferent mechanisms due to local release of neuropeptides, eliciting enhancement of the local microcirculation and inhibition of gastric acid secretion, account for the protective functions that sensory nerves subserve in the gastrointestinal tract (Abdel-Salam et al., 1999; Szolcsányi and Barthó, 2001.). Within the liver, sensory nerve fibers stimulate mechanisms of hepatic protection *via* an efferent release of neurotransmitters.

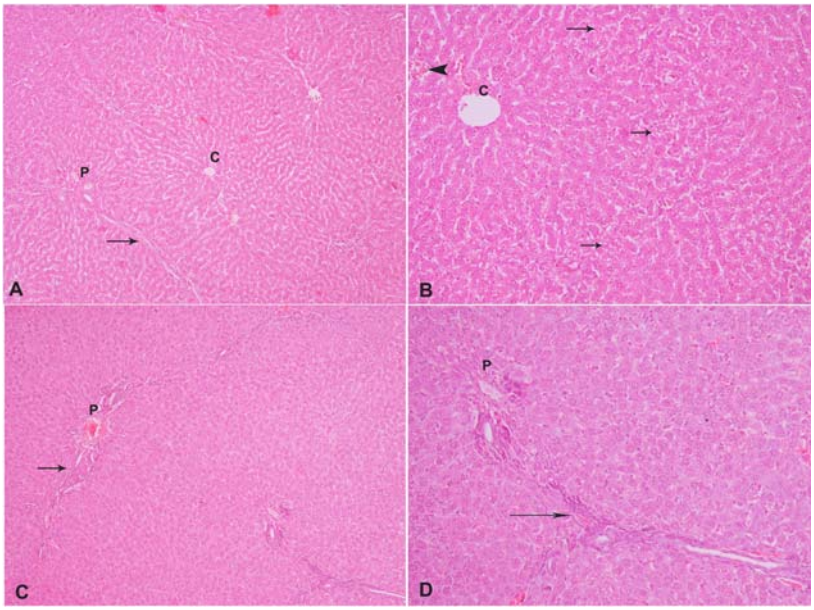

Figure 3: (A) A photomicrograph of a section of liver tissue treated with CCl_4 and aspartame at the dose of 0.625 mg/kg showing the presence of fibrous septa radiating from the portal area within the parenchyma of the hepatic lobule (arrow). Section **(B)** shows necrosis and vacuolar degeneration in the hepatocytes at the boundary of the hepatic lobule (arrow). Sections **(C & D)** show the effect of aspartame at the dose of 1.25 mg/kg, where the fibrosis with mild cellular infiltrate is still present (arrow), but the degeneration and necrosis is markedly reduced.

The rat hepatobiliary tract is densely innervated by CGRP-containing fibers that form dense networks in the fibromuscular layer of the biliary tree and surrounding the portal vein. This suggests the involvement of these peptidergic visceral afferents in regulating hepatobiliary activities, including hemodynamic functions of the hepatic vasculature (Goehler and Sternini, 1996). In this context, it has been shown that orally administered capsaicin protected against the hepatotoxic effects of CCl_4 in rats (Abdel-Salam et al., 2006). Piperine which also activates vanilloid receptors (Szolcsányi, 1983; Liu and Simon, 1996) have been shown to protect against hepatocellular injury and fibrosis caused in rats by bile duct-ligation (Abdel-Salam et al., 2008). It could be that the sensation produced by citric acid or chemical excitation of nociceptors in the stomach by citric acid excite vagal neurons involved in signalling hepatic protection. Citrate absorption is rapid and efficient in man, with 96 to 98 % absorbed within 3 hours (Fegan et al., 1992). Plasma citrate peaked after 32 min, returning to baseline by 90 min (Taylor et al., 1998). Whether citric acid can reach hepatobiliary tract and excite sensory nerve endings or prevent liver injury *via* antioxidant properties is not clear. Increased intracellular citrate synthesis might also be involved. Bjarnason et al. (1992) have shown that the administration of glucose and citrate mitigated the increase in intestinal permeability caused by indomethacin, likely through stimulation of glycolysis and the tricarboxylic acid cycle.

Gustatory stimuli, including bitter- and sour tasting chemicals, trigger cardiovascular and digestive reflexes (Yonemura et al., 1989; Hanamori and Ishiko, 1993; Horio, 2000), and there are pathways from the rostral to the caudal nucleus of the solitary tract that could mediate these actions (Beckman and Whitehead, 1991; Streefland and Jansen, 1999). In rats, sweet taste information induced by a single infusion of sucrose (intra-oral or intra-gastric) or saccharin (intra-oral) results in c-Fos expression in the nucleus of the solitary tract (Streefland et al., 1996). The expression of the immediate early gene c-Fos and subsequent synthesis of its Fos protein, is a marker of neuronal activity (Hunt et al., 1987). Tasting food elicits the release of digestive enzymes, known as cephalic phase of digestion. The non-nutritive sweetener saccharine elicited insulin release prior to increasing plasma glucose levels and sweetness information conducted by chorda tympani appear to provide essential information for eliciting this cephalic phase of insulin release (Tonosaki et al., 2007). Gustatory and other oral sensory signals appear also 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 801-100sec after application (Horio, 2000). Taste cells responding to sweet or bitter taste stimuli secrete the neurotransmitter ATP which is believed to excite primary sensory afferent fibers that convey gustatory signals to the brain. This suggests that there is important information processing and signal coding taking place in the mammalian taste bud after gustatory stimulation (Roper, 2007). It is therefore possible that the observed hepatoprotective effect of citric acid or the sweetener aspartame, 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 solitary neucleus (Whitehead and Frank, 1983) and gustatory cortex (Barnett et al., 1995), suggesting that interactions should occur between taste and other systems.

Citric acid is found in all animal tissues as an intermediate in the Krebs cycle, and therefore no limit has been set on the acceptable daily intake for humans for either the acid or salt (German, 2002). Studies on citrate pharmacokinetics and metabolism in critically ill cirrhotic patients confirmed a major role of hepatic citrate metabolism by demonstrating reduced citrate clearance in

cirrhotic patients, but without citrate-related side effects (Kramer et al., 2003). The acceptable daily intake levels of aspartame established by U.S. Food and Drug Administration and European Food Safety Authority is 50 and 40 mg/kg/day, respectively (Magnuson et al., 2007). In the present study, the doses of aspartame used are notably low. In the body, aspartame is completely hydrolyzed into three metabolites: aspartic acid, phenylalanine, and methanol. All are naturally present in foods and the contribution to the total daily intake of each of these from aspartame is small to trivial (Magnuson et al., 2007). Studies in patients with chronic, alcoholic liver disease, suggested that aspartame in a dose of 15 mg/kg (representing 5 times the average daily intake of adults) did not result in clinical derangements in encephalopathic indices (Hertelendy et al., 1993). Studies in man also indicated that ingestion of dietary phenylalanine, as supplied in a single can of diet cola (providing 184 mg aspartame, of which 104 mg is phenylalanine), is readily handled in both normal and phenylketonuric subjects (Stephanie et al., 1992). Aspartame is not without effects on the central nervous system, but these occur only when high doses of the sweetener are administered. In rats, acute doses of up to 2000 mg/kg, failed to induce significant changes in brain serotonin or dopamine levels (Dailey et al., 1991), but rats given 200 mg/kg of aspartame showed large increments in brain and plasma levels of phenylalanine and its product tyrosine, while mice given aspartame at 13, 130 and 650 mg/kg, have increases of 12, 49 and 47 % respectively in norepinephrine after 3 hours in the hypothalamus and significant increases in norepinephrine in the medulla oblongata and corpus striatum (Coulombe and Sharma, 1986).

In summary, the present study reports the observation that two gustatory stimuli namely citric acid and the sweetening agent aspartame have modulatory effect on the CCl_4-induced hepatic injury in rats. Whether this is the result of an interaction of sour and sweet taste solutions with taste receptor cells on the dorsal surface of the tongue or due to stimulation of upper gastrointestinal mucosal afferents with the activation of neuroendocrine or autonomic reflexes or the result of excitation of sensory nerve endings within the liver remains to be established.

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