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Protective effect of vitamin C against the ethanol mediated toxic effects on human brain glial cells

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Abstract

It is now known that chronic consumption of excessive amounts of alcohol is a major source of social and medical problems. Ethanol-mediated glial cell activation may lead to neuron damage in many ways, including the formation of free radicals and production of pro-inflammatory molecules. Vitamin C (vit-C) is an essential dietary nutrient required as a co-factor for many enzymes and a very efficient antioxidant, protecting cells against free radical-mediated damage. The objective of this study was to evaluate the protective effects of vit-C on glial cell activation and viability against ethanol-mediated toxicity. Human brain astrocyte cells (HA) were exposed to ethanol (0, 50, and 350 mmol/L) for 24 h. We found that glial cells incubated with different concentrations of vit-C increase their vit-C in a dose-dependent manner. HA incubated with 0, 50 or 350 mmol/L of ethanol for up to 24 h showed toxic effects that were proportional to the levels of ethanol in the medium, HA showed increased levels of heat shock protein (Hsp70). However, cells enriched with vit-C before being exposed to ethanol, were better protected against the alcohol-mediated toxicity than non-supplemented cells, and showed significantly lower concentrations of Hsp70. Ethanol also caused increased expression of cyclooxygenase-2 (COX-2) and synthesis of prostaglandin E_2 (PGE_2), which were reduced by vit-C. In summary, HA supplemented with vit-C were significantly more resistant to the ethanol-mediated toxic effects.

Keywords: Glial cells; Ethanol; Cell viability; Vitamin C; Hsp70; COX-2; PGE_2

1. Introduction

Despite the long history and widespread use of ethanol, as well as decades of research into its effects, the mechanism of action remains among the least understood of nearly all psychoactive drugs. Ethanol is the primary choice among mood-altering drugs in the United States. Because ethanol is not only a psychoactive drug but also a food, it is considered part of the basic diet in many cultures. A large intake of ethanol, however, has a deleterious effect on nutritional status. After the ethanol is absorbed, it is distributed to all tissues and fluids of the body in direct proportion to the blood levels. The consumption of ethanol affects the brain more than any other organ. Ethanol-mediate effects in brain are linked to changes in neurotransmitter synthesis (especially the neurotransmitters like serotonin, dopamine, GABA, and glutamate) and alteration of cell membrane fluidity [1-3]. Even though it has been proven that ethanol causes many systemic complications, some researchers are, however, debating about whether a moderate alcohol intake may lower coronary heart disease and other forms of heart failure [4].

Ethanol is implicated in the onset of a variety of immune defects, including the production of pro-inflammatory cytokines and secretion of tumor necrosis factor (TNF-α), interleukine-1β (IL-1β), and interleukine-6 (IL-6) [5, 6]. Recent research indicates that inflammation, oxidative stress, and glial cell activation appear to play key roles in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease [7-12]. Cyclooxygenase (COX) activity has been previously shown to be up-regulated by ethanol in

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astrocytes and neurons, and a nonselective blocker for COX eliminated the ethanol-induced increases of COX activity in both cell types [13]. However, increased COX activity, found only in astrocytes, resulted from an increase in cyclooxygenase-2 (COX-2) expression [13]. In vitro and in vivo studies show that ethanol can alter systemic prostaglandin (PG) concentrations and increases endogenous PG concentrations in the central nervous system (CNS) [14, 15]. Thus, HA appears to be the primary target of ethanol-induced increases in COX-2 expression and synthesis of prostaglandin E_2 (PGE_2) [13].

Hsp70 expression is a useful marker of cellular injury and may help to identify previously unrecognized areas of vulnerability in the nervous system after a neurotoxic stimulus. Hsp70 may also play a neuroprotective role in the brain working as a molecular chaperone to maintain the native conformation of proteins and participating in protein transport, in particular cellular compartments. Hsp70 is expressed constitutively in very low levels in human cells, but its expression abundantly increases in response to various physiological stressors, including exposure to heat shock, inflammatory stimulus, and ethanol. Changes in the redox status have been suggested to mediate the induction of Hsp70 that follows exposure to oxidizing agents such as ethanol [16]. Expression is regulated at both transcriptional and translational levels. Different studies have shown in the animal model that ethanol intake induces the formation of protein carbonyls and Hsp70 expression in all brain regions [16, 17]. Chronic human maternal ethanol consumption has been shown to result in increased Hsp70 levels in different regions of the developing brain. Moreover, in vitro incubation of primary astroglial cultures and astrocytes with ethanol has shown an increased Hsp70 content [17].

Ascorbic acid concentration in brain is highly regulated. The brain normally contains high concentrations of vit-C, which is actively taken through the choroids plexus. However, its specific functions in the CNS are only beginning to be elucidated. Vit-C acts as part of the intracellular antioxidant network, and as such is an important neuroprotective constituent. Growing evidence indicates that vit-C also acts as a neuromodulator. Recently, it has become clear that antioxidant nutrients, including vit-C, are important for neurological function [18-22]. High intake of vitamin E and C has been found to be associated with lower risk of Alzheimer's disease [8]. Therefore, the objective of this study was to assess the protective effect of physiological concentrations of vit-C against the ethanol mediated toxic effects and PGE_2 production in HA relevant to neurological damage.

2. Methods and materials

2.1. Cell culture

HA cells were obtained from Clonetics Laboratories (BioWhittaker, Inc. Walkersville, MD). Cells were cultured in neurobasal medium (Gibco Laboratories, Life Technologies, Gaithersburg, MD) under humidified 5% CO_2. The medium was supplemented with 8% fetal bovine serum, 20 ng/mL human recombinant growth factor, 6 μg/mL transferrin, 1.4 μg/mL insulin, 50 U/mL penicillin/streptomycin and 0.6 μg/mL amphotericin B (Sigma, St. Louis, MO). Cells were plated in 1% gelatin-coated flasks or six-well plates (Corning Inc., Corning, NY) and the medium was changed every two days until they reached confluence. Cells of the fourth to seventh passage were used for the experiments. All cells were tested for HIV-1, hepatitis B and C, mycoplasm, bacteria, yeast and fungi. Cell viability was determined by the MTT test [23]. Cell viability was not altered during incubation with different concentrations of vit-C. To deprive cells from serum, plates were washed with Hanks' balanced salt solution (Gibco Laboratories, Life Technologies, Gaithersburg, MD) and fresh medium without serum was added. We did not detect any changes in cell viability or apoptotic manifestation in HA cells after removal of serum. Aspirin treatment was performed by preincubating cells for 1 h with a final concentration of aspirin of 500 μmol/L in a serum free medium. HeLa cell lysate (heat shocked) (Stressgen, Victoria, BC, Canada) was used as an internal positive control. The HeLa cell line was originally derived from epitheloid carcinoma tissue (cervix) from a human donor.

2.2. MTT test

The [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) test was used to evaluate the ethanol-mediated cytotoxic effect on HA following exposure to ethanol. Confluent cells grown in 96-well plates were treated with different concentrations of ethanol (0, 50, and 350 mmol/L). MTT (Sigma, St. Louis, MO) was dissolved in Hanks' balanced salt solution to obtain a final concentration of 5 mg/mL, and used freshly or stored at −20°C (stock solution is stable at this temperature). 10 μL of MTT solution were added to each well using a six-channel pipette to reach a final MTT concentration of 0.5 mg/mL. Two sets of controls (untreated cells) on both sides of the plate were used. Differences between left and right control indicate the influence of seeding, humidity and/or temperature on the obtained results. Cells were incubated for 4 h, then medium removed after incubation (plates were inverted to rapidly remove the medium), plates were gently tapped on paper tissue to complete medium removal. Formazan dissolved in organic solvent was added and plates placed on a shaker and agitated for 10 to 20 min. Plates were read at 550 to 570 nm (L1) and 620 to 650 nm (L2) on scanning microplate reader spectrophotometer; 620 to 650 nm absorbance results from cell debris and well imperfections. Final optical density (OD) obtained OD = L1-L2 was used to calculate the percentage of cell survival and expressed as: (absorbance treated wells/absorbance control wells) x 100%.

2.3. Vitamin C quantification

Intracellular levels of vit-C were measured by paired-ion reversed-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection as described before [24]. In brief, one volume of cellular homogenate was mixed with one volume of cold 5% (w/v) metaphosphoric acid, 1 mmol/L diethylenetriaminepentaacetic acid, and centrifuged to remove the precipitated proteins. An aliquot of the supernatant was subjected to chromatography on a LC8 column (150 mm x 4.6 mm i.d., 3 μm particle size) (Supelco, Bellefonte, PA) using 99% deionized water and 1% methanol containing 40 mmol/L sodium acetate and 1.5 mmol/L dodecyltriethylammonium phosphate (Q12 ion pair cocktail, Regis, Morton Grove, IL) as the mobile phase. Samples were injected with an autosampler, HP 1100 series (Hewlett Packard). Vit-C was detected at an applied potential of +0.6 V by a LC 4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Vit-C was eluted as a single peak with a retention time of 5.5 min. Peaks were integrated with a ChemStation, (Hewlett Packard). Vit-C concentration was calculated based on a calibration curve, and its concentration was expressed in nmol/mg protein.

2.4. Protein determination

Protein concentration in the cell extract was determined by the Lowry method [25] using bovine serum albumin as a standard.

2.5. Heat shock protein 70 (Hsp70) and cyclooxygenase-2 (COX-2) quantification

Hsp70 and COX-2 were measured as reported previously [26, 27] using a western blotting technique. Samples are typically unpurified and contain several different proteins that prevent any specific measurement of a single protein; therefore, they are separated by gel electrophoresis. After samples were run and proteins separated using a SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gel bands were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) using a Mini-Trans-Blot SD wet transfer cell (Bio-Rad, Richmond, VA). Immunoblotting of the PVDF membranes was performed following standard procedures [28]. The protein recognized by the specific monoclonal antibody against the Hsp70 and COX-2, and following incubation with the secondary antibody-HRP against the mouse IgG, was visualized by chemiluminescence methods (Renaissance, NEN-Life Science Products, Boston, MA). Densitometric quantification of the immunoblotted membranes was performed with an Image Analyzer System (Inotech S-100, Sunnyvale, CA).

2.6. Prostaglandin E_2 (PGE_2) determination

PGE_2 was measured using a high sensitivity immunoassay based in a competitive binding technique in which prostaglandins present in a sample compete with a fixed amount of alkaline phosphatase-labeled PG for sites on a mouse monoclonal antibody. During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate wells. Following a wash to remove excess conjugate and unbound sample, a substrate solution was added to the wells to determine the bound enzyme activity. The intensity of the color, therefore, was inversely proportional to the concentration of the PGE_2 measured in the sample [29, 30].

2.7. Statistical analysis

Repeated measures analysis of variance (ANOVA) followed by Tukey's HSD for multiple comparisons to assess the significance of differences between treatments were performed by using Systat 10 program (SPSS, Chicago, IL). Data are presented as mean ± SEM with $P < 0.05$ considered significant and denoted by letters.

3. Results

3.1. Toxic effect of ethanol on HA

A series of preliminary studies examining the toxic effects of ethanol on HA cells were carried out by exposing HA to different concentrations of ethanol (0, 50, 150, 350 mmol/L), and during different time intervals (0, 12, 24, and 48 h). From these preliminary studies, we selected 50 and 350 mmol/L ethanol concentrations and 24 h as the optimum conditions to carry out the final experiments reported here. These concentrations of ethanol were capable of inducing significant toxic effects to HA cells, decreasing their cell viability and increasing Hsp70 expression as well as PGE_2 synthesis, compared with the untreated control cells group. These concentrations are representative of low degree ethanol intoxication with confusion and disturbances of sensation (50 mmol/L), and a high degree of intoxication and death (350 mmol/L). The later concentration was used to induce an accelerated acute in vitro ethanol insult to mimic in vivo effects of high ethanol consumption.

3.2. Incorporation of vitamin C into HA cells in culture

The uptake of vit-C by HA cells in culture was measured first. The HA used in our experiments–like most of the cells used in in vitro experiments–contain very low concentrations of endogenous vit-C (0.5 ± 0.3 nmol/mg protein). These low intracellular levels of vit-C are a consequence of the low concentration of vit-C present in the culture medium, which in general contains only about 1 to 5 μmol/L

Table 1
Ascorbic acid uptake by human astrocyte cells incubated with various concentrations of vitamin C for 8 h[1]

Vitamin C (μmol/L)	Intracellular vitamin C (nmol/mg protein)
50	1.9 ± 0.1[a]
100	5.7 ± 0.2[b]
150	10.9 ± 0.7[c]
250	16.7 ± 0.8[d]
350	23.3 ± 1.5[e]

[1] Values are means ± SEM, $n = 3$, with 6 plates in each experiment. Means not sharing a superscript letter differ. $P < 0.05$, based on repeated measures ANOVA (Tukey's test).

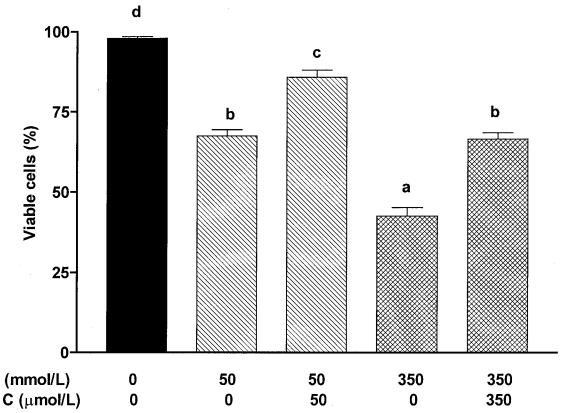

Fig. 1. Effect of vit-C on human astrocyte cells viability following exposure to ethanol for 24 h. Each column represents the mean of three independent experiments (*n* 3), with 6 plates in each experiment. Bars not sharing a superscript letter differ, $P < 0.05$, based on repeated measures ANOVA (Tukey's test).

[31]. However, incubation of HA in neurobasal medium containing 150 μmol/L of vit-C led to a time-dependent accumulation of vit-C inside the cells. Intracellular levels of vit-C reached a maximum of 10.9 ± 0.7 nmol/mg protein after 8 h of incubation. Using this information, we incubated the cells for 8 h to obtain maximum vit-C incorporation by the cells. We found that cells accumulated vit-C in a dose-dependent manner with maximum incorporation of about 23 nmol/mg protein when cells were incubated with 350 μmol/L of vit-C (Table 1).

3.3. Effect of vitamin C on HA cell viability following exposure to ethanol during 24 h

Treatment of HA cells with different concentrations of ethanol (25 to 350 mol/L) caused a dose response effect on cell viability. Ethanol concentration above 150 mmol/L caused strong detrimental effects. Based on these experiments we selected a low, and a high, concentrations of ethanol 50 and 350 mmol/L, as representative of ethanol intoxication (50 mmol/L), and the high concentration (350 mmol/L) to mimic the long-term effects of high ethanol intake in this acute cell model. HA cells exposed to ethanol showed a dose-effect decrease in cell viability after 24 h incubation (Fig. 1). These effects were significantly more marked in cells treated with 350 mmol/L compared to cells exposed to 50 mmol/L (Fig. 1).

The amount of ethanol in the bloodstream is referred to as Blood Alcohol Level. It is recorded in milligrams of ethanol per 100 mL of blood, or milligrams percentage. When ethanol is consumed, its effects are felt quickly because 20% goes directly from the stomach into the bloodstream. 0.1% of ethanol in blood is defined as legal intoxication by most states, even though at this level of ethanol, people look normal by ordinary observations. Between 0.1% and 0.3% disturbances of sensations and slurred speech appear, and after 0.5% complete unconsciousness and depressed reflexes appear. The two concentrations of ethanol selected in these experiments were carefully selected to evaluate the effect of ethanol on cell cytotoxicity and to assess the role of vit-C on protecting cells against ethanol-mediated toxic effects. Cells supplemented with vit-C (50 μmol/L) for 8 h previously being exposed to ethanol (50 mmol/L) for 24 h, showed a percentage of cell viability of 85.8 ± 2.2 compared to 67.5 ± 2.0 found in the non-supplemented group ($P = 0.001$). When cells were supplemented with vit-C (350 μmol/L) for 8 h prior to being exposed to ethanol (350 mmol/L) for 24 h, those cells enriched with vit-C were significantly ($P < 0001$) protected (66.5 ± 2.0) compared with non-supplemented cells (42.5 ± 2.7) (Fig. 1**)**.

3.4. Effect of ethanol on Hsp70 expression in primary astrocyte cultures

Following incubation with 0, 50, and 350 mmol/L of ethanol for 24 h, cell activation was determined by measuring heat shock protein 70 (Hsp70). Following incubation, cells were collected and protein extracted for western blotting analysis and determination of the protein Hsp70 (Fig. 2).

Increased levels of Hsp70 were observed when cells were exposed to ethanol (50 and 350 mmol/L) for 24 h. Hsp70 expression increased by 42% and 184% following ethanol exposure (50 and 350 mmol/L) compared to control non-treated cells (Fig. 2). However, when cells were supplemented with vit-C for 8 h prior to being exposed to ethanol, cells were more resistant to the toxic effects mediated by the different levels of ethanol present in the media (Fig. 3) . Cells supplemented with 150 μmol/L vit-C before being exposed to 350 mmol/L ethanol showed a 3% reduction, however, when cells were supplemented with 350 μmol/L vit-C a 62% reduction in the expression was observed (Fig. 3). Similarly, when cells were pretreated with aspirin (500 μmol/L) for 1 h before being exposed to 350 mmol/L ethanol a 44% reduction in Hsp70 was detected (Fig. 3).

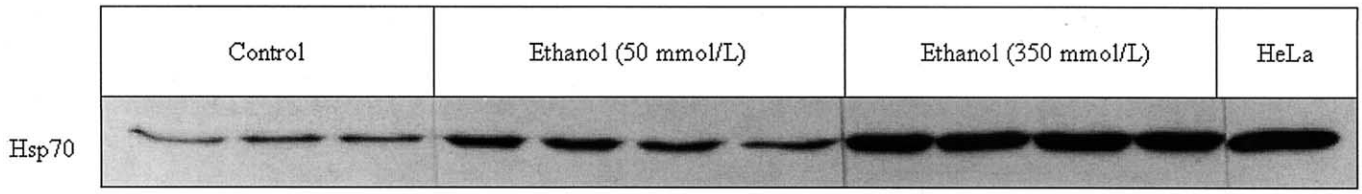

Fig. 2. Western blotting analysis of Hsp70 protein expression (molecular weight 70kDa) in human astrocyte cells exposed to ethanol (50 and 350 mmol/L) for 24 h. Each band represents three independent experiments, with triplicates in each experiment.

3.5. Effect of ethanol on COX-2 expression in primary astrocyte cultures

Following incubation with 0, 50, and 350 mmol/L of ethanol for 24 h, COX-2 expression was determined by western blotting analysis. A significant elevated expression was detected when cells were exposed to ethanol (350 mmol/L) for 24 h (Fig. 4). When cells were supplemented with vit-C (150 and 350 μmol/L) for 8 h prior to being exposed to ethanol (350 mmol/L) for 24 h, a reduction in COX-2 expression was observed. However, cells pretreated with aspirin (500 μmol/L) for 1 h before the exposure to ethanol, although they synthesized much lower concentration of PGE_2, (Table 2) showed the strongest expression of COX-2 (Fig. 4). Perhaps, changes in PGE_2 concentration mediated by aspirin, is a very strong modulatory effect of COX-2 expression. Prostaglandins are generated through the release of arachidonic acid (AA) by the action of cytosolic phospholipase A2 (cPLA2) and the consequent catalytic conversion of AA to PGE_2 by COX, however, we did not observe changes in cPLA2 expression following incubation with ethanol (data not shown). These findings are in accordance with a previous report by Luo et al. [13] who also did not find changes either in the activity or in the expression of cPLA2 following exposure to ethanol.

3.6. Effect of ethanol on PGE_2 content

Following incubation with 0, 50, and 350 mmol/L of ethanol for 24 h, PGE_2 levels were measured in the medium by using a high sensitivity immunoassay. Significant elevated PGE_2 levels (294.8 $\pm$ 2.8 pg/mL) were detected when cells were exposed to ethanol (350 mmol/L) for 24 h (Table 2). Interestingly, when cells were supplemented with vit-C (150 and 350 μmol/L) for 8 h prior to being exposed to ethanol (350 mmol/L) for 24 h, a significant ($P < 0.003$) reduction in PGE_2 levels was detected (271.3 $\pm$ 2.3 pg/mL and 273.3 $\pm$ 3.5 pg/mL, respectively). In addition, cells pretreated with aspirin (500 μmol/L) for 1 h before the exposure to ethanol showed a significant ($P < 0.001$) reduction in PGE_2 levels (219.7 $\pm$ 1.2 pg/mL), even lower than the control cells.

4. Discussion

The results of this study demonstrate that cultured primary HA cells respond robustly to the effects of ethanol with a concentration-dependent increase in cell cytotoxicity, decreased cell viability and increase in Hsp70. In fact, when cells were incubated with different concentrations of ethanol for 24 h, cells experienced significant toxic effects and

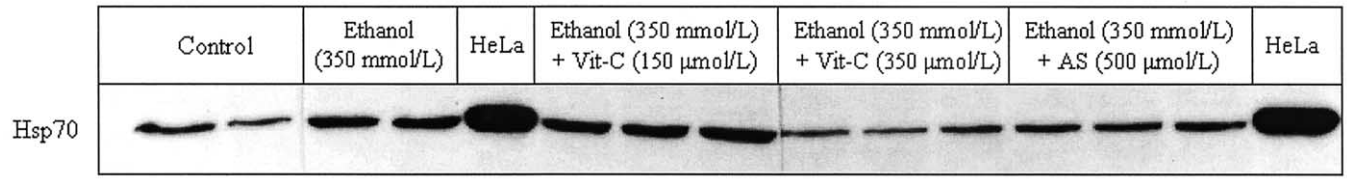

Fig. 3. Western blotting analysis of Hsp70 protein expression (molecular weight 70kDa) in human astrocyte cells supplemented with vit-C (150 or 350 μmol/L) for 8 h, or pretreated with aspirin (500 μmol/L) for 1 h, prior to being exposed to 350 mmol/L of ethanol for 24 h. Each band represents three independent experiments, with triplicates in each experiment.

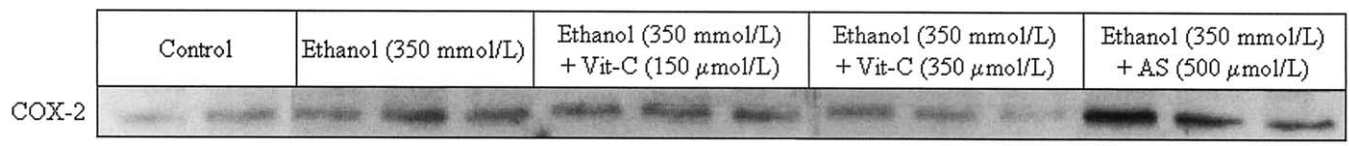

Fig. 4. Western blotting analysis of COX-2 protein expression (molecular weight 70kDa), in human astrocyte cells supplemented with vit-C (150 or 350 μmol/L) for 8 h, or pretreated with aspirin (500 μmol/L) for 1 h, prior to being exposed to 350 mmol/L of ethanol for 24 h. Each band represents three independent experiments, with triplicates in each experiment.

Table 2
Effect of vitamin C and aspirin on levels of PGE_2 after human astrocytes were exposed to ethanol for 24 h[1]

	PGE_2 (pg/mL)
Control	249.9 ± 3.4^b
Ethanol (350 mmol/L)	294.8 ± 2.8^d
Ethanol (350 mmol/L) + Vitamin C (150 μmol/L)	271.3 ± 2.3^c
Ethanol (350 mmol/L) + Vitamin C (350 μmol/L)	273.3 ± 3.5^c
Ethanol (350 mmol/L) + Aspirin (500 μmol/L)	219.7 ± 1.2^a

[1] Values are means ± SEM, $n = 3$, with 6 plates in each experiment. Means not sharing a superscript letter differ. $P < 0.05$, based on repeated measures ANOVA (Tukey's test).

a dose-dependent decrease in the cell viability as the MTT test indicates (Fig. 1). In addition to the toxic effects reported, an ethanol-mediated inflammatory response was observed. Moderate alcohol consumption, however, has been associated with a decreased risk for ischemic heart disease [32].

The presence of ethanol caused a strong cell response in HA with detrimental effects on cell viability and changes in the expression of Hsp70 and COX-2. This suggests that high ethanol intake may contribute to the inflammatory processes in brain. Increasing evidence indicates that inflammation is involved in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease.

In recent years, different studies have demonstrated that cells increase production of proteins when their environment changes. Heat shock proteins are synthesized under different kind of stress conditions, like inflammation, infection, ischemia and exposure of the cell to toxins or malignant transformation [17, 33]. Hsp70 is expressed at high levels in stress conditions. Hsp70 participates in translation, protein translocation, proteolysis and protein folding, suppressing aggregation and reactivating denatured proteins [33]. When we exposed primary astrocyte cell cultures to ethanol, we observed a significant increase in Hsp70 content in the cells, supporting previous studies [13, 34, 35]. Interestingly, changes in the Hsp70 expression were smaller in those cells previously enriched with vit-C before being exposed to ethanol, indicating that cells were able to cope better with the ethanol-induced insult.

A significant body of evidence shows that Hsp70 levels increase in cells exposed to toxic compounds, therefore the assessment of Hsp70 and cell viability in combination provide a more accurate information to assess the ethanol toxicity effects in brain glial cells and evaluate the protective effect of vit-C against ethanol-mediated deleterious effects.

Ethanol exposure resulted on increased prostaglandins synthesis, which are important mediators of localized inflammation, particularly PGE_2. Prostaglandin synthesis occurs first by the release of AA from the membrane through the action of cPLA2 on membrane-bound phospholipids. A process completed by the enzyme cyclooxygenase via conversion of AA to PGE_2. Cyclooxygenase-1 (COX-1) is the constitutive form of the enzyme while COX-2 is the inducible form. Our results confirm an increased expression of COX-2 and synthesis of PGE_2 during ethanol exposure. Significant body of evidence has shown that nonsteroidal anti-inflammatory drugs (NSAID) effectively inhibit PG synthesis and decrease inflammation [36]. In fact, NSAID and COX-2 inhibitors have long been known to have a beneficial effect in inflammatory diseases like rheumatoid arthritis, [36] and various epidemiological studies have now demonstrated that the use of NSAID is associated with a reduced risk of developing dementia [37, 38].

We have shown that HA exposed to ethanol responded with a significant increase in PGE_2 synthesis, which were ameliorated or prevented by vit-C and aspirin. However, we did not observe changes in cPLA2 expression following treatment with ethanol (data not shown), indicating that the main player in the regulation of PGE_2 appears to be COX-2. Supporting these findings, other studies have also shown that neither the activity nor the expression of cPLA2 was affected by ethanol [13].

There is now increasing evidence that elevated expression of COX-2 plays a role in development and progression of certain pathological processes. In fact, increased levels of prostanoids have been implicated in various neuropathological diseases, although little is known about their cellular sources inside the brain [39]. COX-2 is readily up-regulated in astrocyte and microglial cell cultures treated with pro-inflammatory cytokines, lipopolysaccharide, and believed to contribute to CNS inflammatory processes [40, 41]. After ischemia-reperfusion, reactive and hypertrophic astrocytes may be the major sources of prostaglandins in the human brain [41]. Immunocytochemical methods have shown that cPLA2 is distributed throughout the brain [42]. Interestingly, the distribution of cPLA2 coincides with that of protein kinase C in the rat brain, and suggests that these enzymes play a central role in neurotransmitter release and long-term potentiation under normal conditions [42].

As described in the results section, HA cells incubated with different concentrations of vit-C increased their vit-C concentration in a dose-dependent manner, and were significantly more resistant to the ethanol-mediated toxic effects compared with non-supplemented cells. Interestingly, cells supplemented with 350 μmol/L vit-C or treated with 500 μmol/L aspirin provided cells with a significant protective effect reduction Hsp70 expression by 62% and 44% respectively (Fig. 3). The specific mechanisms of the protective effects remain unclear, although we believe that the down-regulation of COX-2 expression and decreased synthesis of PGE_2 may have contributed to the observed effects. Vit-C may have protected cells by enhancing the activity of free radical scavenging enzymes like superoxide dismutase (SOD) and catalase. In fact, in a rat model of alcohol-induced toxicity, co-administration of vit-C with alcohol clearly showed a protective action maintaining SOD and catalase activities and in reducing ethanol induced toxicity [43]. Thus, a generous intake of fruits and vegetables rich in

vit-C will contribute to increasing the levels of vit-C in the brain as well as preserving brain cells' function against the ethanol-induced toxicity.

The nervous system, with its long-lived neurons, is vitally dependent on an effective nutritional environment and removal of any damaged protein. Optimum metabolic and macrophagic functions of the glial cells in the CNS appear essential for the proper function of the nervous system itself to degrade modified proteins, [31] probably increased during cytotoxic processes induced by ethanol. In addition, activation of the glial cells following exposure to a toxic compound may initiate a strong inflammatory cascade with detrimental effects upon the CNS and progressive cognitive decline. We, and others, have demonstrated that ethanol up-regulates expression of COX-2 and synthesis of PGE_2 relevant to inflammation. However, it is necessary to better understand the effects of ethanol in the brain to evaluate how the ethanol may affect glial cell function and contribute to the initiation of an inflammatory response with significant detrimental effects to the CNS. Further research is needed to evaluate the role of ethanol on astrocyte cell's expression of cytokines, perhaps relevant to neurodegenerative diseases.

One limitation of this study is that this is an in vitro design, and even though the cells are primary HA, the important implications on neurotransmitter synthesis and its implications on behavior changes induced by ethanol could not be addressed in this study. Examining the potential benefits of vit-C on brain cell function and neurotransmitter levels and behavior deserves more attention.

In summary, ethanol-mediated toxic effects in brain cells appear to be dose-dependent, affecting cell viability and altering several intracellular pathways leading to increased expression of molecules associated with stress and inflammation. HA having higher intracellular concentrations of vit-C appear to be better protected against ethanol mediated deleterious effects.

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