DOI: 10.2478/10004-1254-60-2009-1927

Scientific Paper

COMPARISON OF ETHANOL AND ACETALDEHYDE TOXICITY IN RAT ASTROCYTES IN PRIMARY CULTURE

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Received in November 2008
Accepted in April 2009

This study compared the effects of toxicity of ethanol and its first metabolite acetaldehyde in rat astrocytes through cell viability and cell proliferation. The cells were treated with different concentrations of ethanol in the presence or absence of a catalase inhibitor 2-amino-1,2,4 triazole (AMT) or with different concentrations of acetaldehyde. Cell viability was assessed using the trypan blue test. Cell proliferation was assessed after 24 hours and after seven days of exposure to either ethanol or acetaldehyde.

We showed that both ethanol and acetaldehyde decreased cell viability in a dose-dependent manner. In proliferation studies, after seven days of exposure to either ethanol or acetaldehyde, we observed a significant dose-dependent decrease in cell number. The protein content study showed biphasic dose-response curves, after 24 hours and seven days of exposure to either ethanol or acetaldehyde. Co-incubation in the presence of AMT significantly reduced the inhibitory effect of ethanol on cell proliferation.

We concluded that long-term exposure of astrocytes to ethanol is more toxic than acute exposure. Acetaldehyde is a much more potent toxin than ethanol, and at least a part of ethanol toxicity is due to ethanol's first metabolite acetaldehyde.

KEY WORDS: *2-amino-1,2,4 triazole, AMT, cell proliferation, cell viability, trypan blue*

Ethanol consumption has long been associated with brain damage. Numerous experimental studies and necropsy examinations of chronic alcoholics have shown a wide range of structural and functional alterations in neurons and astrocytes (1-3). Such alterations are also seen in children with the foetal alcohol syndrome (4). Astrocytes are the major brain cell population; they play an important role in guiding migrating neurons during development, in regulating neurotransmitter and ion levels, in neuron nutrition, and in production of neurotrophic factors (5). Astrocytes are also a major site for bioactivation and detoxification of neurotoxins (6). Many studies have shown that both ethanol and acetaldehyde disturb astroglial growth and differentiation; however, the mechanism remained elusive (7-11). In addition, a number of ethanol effects, including psychopharmacological and neurotoxic effects, are believed to be mediated through its first metabolite, acetaldehyde (12-14).

Acetaldehyde, derived from peripheral metabolism of ethanol, crosses the blood-brain barrier with difficulty due to alcohol- and aldehyde-dehydrogenase (15). The localisation and relevance of ethanol metabolism in the brain is still controversial. The adult mammalian brain contains three enzyme systems for oxidising ethanol to acetaldehyde: cytochrome P450 (CYP) 2E1, catalase, and alcohol-dehydrogenase (16). Studies of naive rat brain have established a crucial role of catalase in oxidising ethanol and forming acetaldehyde. In addition, no evidence of alcohol dehydrogenase or CYP2E1 in this process was found. Metyrapone (CYP inhibitor) or pyrazole

(alcohol-dehydrogenase inhibitor) did not affect the formation of acetaldehyde from ethanol in rat brain homogenates; at the same time, the presence of catalase inhibitors 2-amino-1,2,4 triazole (AMT), cyanamide, or sodium azide lowered acetaldehyde formation in a dose-dependent manner (17-20). The enzyme activity is localised exclusively in the microperoxisomes of aminergic neurone perikaryons and in glial cells (21). Many pieces of data support the notion that acetaldehyde is endowed with positive reinforcing properties, which play a crucial role in mediating ethanol euphoria and pave the way for alcohol craving (22). Animals pre-treated with catalase inhibitor AMT showed shorter ethanol narcosis period and lower mortality (23), less locomotor depression (24), blockade of ethanol-induced taste aversion and reduction of ethanol intake (25). The brain catalase system seems to play a role in the development of tolerance to the hypnotic effect of ethanol, but it does not alter tolerance to the hypothermic or metabolic effects (26).

There is evidence that astrocytes may respond to different injuries by altering phenotype, which involves upregulation of a large number of molecules (27). It was reported that astrocytes in primary cultures respond to a range of neurotoxic compounds with a biphasic dose-response; response increases at low, subtoxic doses, and is followed by a decrease at higher, cytotoxic doses (28).

Literature reports separate data for ethanol and acetaldehyde effects on astrocyte viability and proliferation, obtained with different methods. There are no data if ethanol or acetaldehyde provoke biphasic response in primary astrocyte cultures.

The aim of this study was to compare the effect of ethanol and acetaldehyde toxicity on rat cortical astrocytes in primary culture by determining cell viability and cell proliferation. Our secondary goal was to establish the role of acetaldehyde in ethanol toxicity in cultured astrocytes.

MATERIALS AND METHODS

Materials

L-15 Leibowitz medium, foetal bovine serum (FBS), Dulbecco's modified Eagle medium and Ham's nutrient mixture F-12) (DMEM / F12), penicillin-streptomycin (10,000 IU mL^{-1} - 10,000 UG mL^{-1}) (P/S), and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL, Life Technologies, Paisley, Scotland. Ethanol and acetaldehyde were from Merck, Darmstadt, Germany. AMT, staurosporine, trypan blue and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, USA. Bio-Rad protein assay was obtained from Bio-Rad Laboratories, Munich, Germany.

Animals

Newborn Wistar rats (postnatal day 2) were obtained from our own breeding colony. The animals were maintained under constant environmental conditions, with an ambient temperature of (22±1) °C, relative humidity (55±10) %, and a natural light-dark cycle. The breeding colony was kept in Ehret type 4 cages (Germany); the bedding material was Lignocel 3/4. The colony received standard rodent diet (Altormin, Germany), and had free access to food and water. We used four newborn animals for each experiment.

All animal procedures were approved by the National Animal Ethical Committee of the Republic of Slovenia (licence number 323-02-232/2005/2) and were conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

Preparation of astrocyte cultures

Primary cultures of rat cortical astrocytes were prepared from the brain of newborn Wistar rats. Newborn rats (postnatal day 2) were decapitated and the brains removed aseptically.

After removal of meninges, cortices were transferred to a Petri dish containing L-15 (Leibowitz) medium. The cortices were then mechanically dissociated into 10 mL of culture medium consisting of DMEM/F12 (1:1), 10 % FBS, 100 U mL^{-1}penicillin, and 100 μg mL^{-1} streptomycin. Cell suspension was triturated and plated onto 35 mmol L^{-1} ($2x10^6$ cells per dish). Cells were grown at 37 °C in a water-saturated air environment containing 10 % CO_2 until they became confluent (10 to 12 days). Culture medium was changed every 48 hours after plating. The cultures were used for treatment with either ethanol or acetaldehyde.

The purity of our culture was checked using immunocytochemical staining for glial fibrillary acidic protein, which is the major component of astrocyte

cytoskeleton. The staining indicated that more than 90 % of the cells in our experimental model were astrocytes, which repeats our previous purity results (29).

Treatment of the cells

To determine toxic effects of ethanol and acetaldehyde after 24 hours of exposure, the cells were treated as follows: after the cultures became confluent, culture medium was replaced with 1 mL of fresh serum-free medium and the cells were treated with different concentrations of either ethanol (50 mmol L^{-1} to 1200 mmol L^{-1}) or acetaldehyde (0.25 mmol L^{-1} to 150 mmol L^{-1}), for 24 hours. In another set of experiments, the cells were pre-treated with catalase inhibitor AMT (10 mmol L^{-1}) for six hours, and then treated with different concentrations of ethanol (50 mmol L^{-1} to 1200 mmol L^{-1}) for 24 hours.

Ethanol and acetaldehyde effects after seven days of exposure were determined as follows: after the first medium changing (3rd day after plating) the cells were grown in a medium containing different concentrations of either ethanol (50 mmol L^{-1} to 1200 mmol L^{-1}) in the presence or absence of AMT (10 mmol L^{-1}) or acetaldehyde (0.25 mmol L^{-1} to 150 mmol L^{-1}) for the next seven days. The medium was changed every 48 hours.

Control cells were grown under the same conditions, but in the absence of ethanol or acetaldehyde, or were treated with 10 mmol L^{-1} AMT only.

After the treatment, cells from separate dishes were used for viability test, cell counting, or protein determination.

Determination of cell viability

Cell viability was assessed using the trypan blue test according to a modified method of Uliasz and Hewett (30) after having treated the cells with different concentrations of either ethanol or acetaldehyde for 24 hours. Positive control cells were treated with 1 µmol L^{-1} staurosporine.

After the treatment, the culture medium was replaced by 0.1 mL of 0.4 % trypan blue; after 1 minute of staining, the cultures were rinsed with PBS (pH 7.2). Cells excluding the stain were considered viable. The number of trypan blue-stained cells and trypan blue-free cells was counted using a light microscope at 100 x magnification. The total number of cells scored per experimental point was 100.

Determination of cell proliferation

Cell proliferation was assessed by cell counting and protein determination after treatment with different concentrations of either ethanol or acetaldehyde for 24 hours or seven days.

Cells were counted after treatment and removal of culture medium using a light microscope at 100 x magnification (31). The total number of cells scored per experimental point was 100.

Proteins were also determined after treatment and removal of culture medium, in cells harvested from individual dishes, according to the method of Bradford (32) using bovine serum albumin as a standard.

Statistical analysis

The results are shown as mean ± standard error of mean (SEM) of three independent determinations. One-way ANOVA with Tukey's *post hoc* test were employed to calculate the significance of differences between the means. A p-value of <0.05 was considered statistically significant.

RESULTS

The effect of ethanol and acetaldehyde on cell viability

Incubation of the cultured astrocytes in the presence of ethanol for 24 hours did not influence cell viability until ethanol reached concentration of 700 mmol L^{-1}. Estimated EC_{50} was 847.9 mmol L^{-1}. Pre-treatment with AMT did not diminish the toxic effect of ethanol (Figure 1A). Acetaldehyde showed much higher toxicity than ethanol; cell viability was affected at 25 mmol L^{-1} of acetaldehyde, with EC_{50} of 47.2 mmol L^{-1} (Figure 1B).

The effect of ethanol and acetaldehyde on cell proliferation

Below the concentration 700 mmol L^{-1}, acute exposure of the cultured astrocytes to ethanol for 24 hours did not affect the number of cells in the culture, either in the presence or absence of AMT (Figure 2A). Similarly, we did not observe any decrease in cell number after acute exposure to acetaldehyde until the cut-off concentration of 50 mmol L^{-1} (Figure 2B). Protein content showed biphasic effect of ethanol (Figure 2C) and acetaldehyde (Figure 2D) after 24 hours of exposure. Low concentrations stimulated

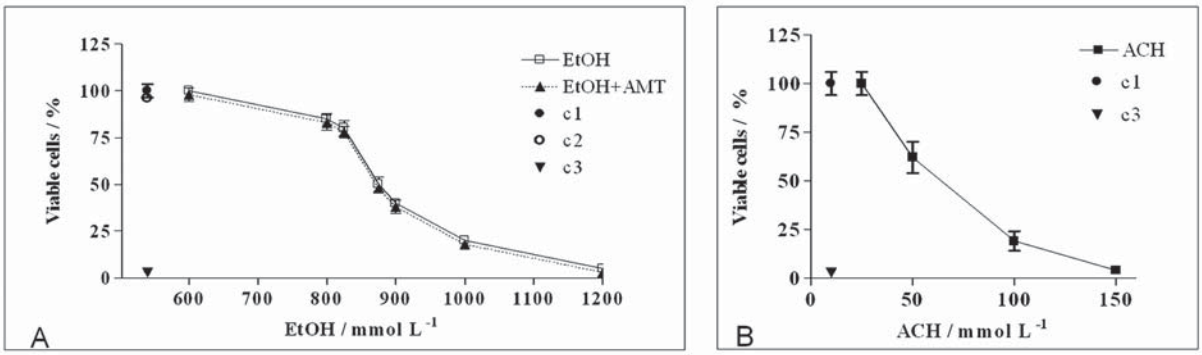

Figure 1 *Effect of ethanol (EtOH) at different concentrations in the presence or absence of catalase inhibitor AMT (10 mmol L^{-1}) (A) and acetaldehyde (ACH) (B) on the viability of cultured astrocytes after 24 hours of exposure. Each point is the mean ± SEM of three independent determinations. c1, control cells - growth medium; c2, control cells, treated with 10 mmol L^{-1} AMT; c3, control cells, treated with 1 µmol L^{-1} staurosporine.*

protein production, but it was inhibited at higher concentrations of either compound. Pre-incubation of the cells with AMT cancelled the biphasic effect of ethanol on protein production, and shifted the inhibitory effect to higher ethanol concentrations (Figure 2C).

Chronic exposure of growing cells to either ethanol or acetaldehyde significantly decreased cell number in the cultures. The first significant decrease in cell number versus control cells after seven days of exposure was observed at 200 mmol L^{-1} of ethanol, with an estimated EC_{50} of 240 mmol L^{-1}.

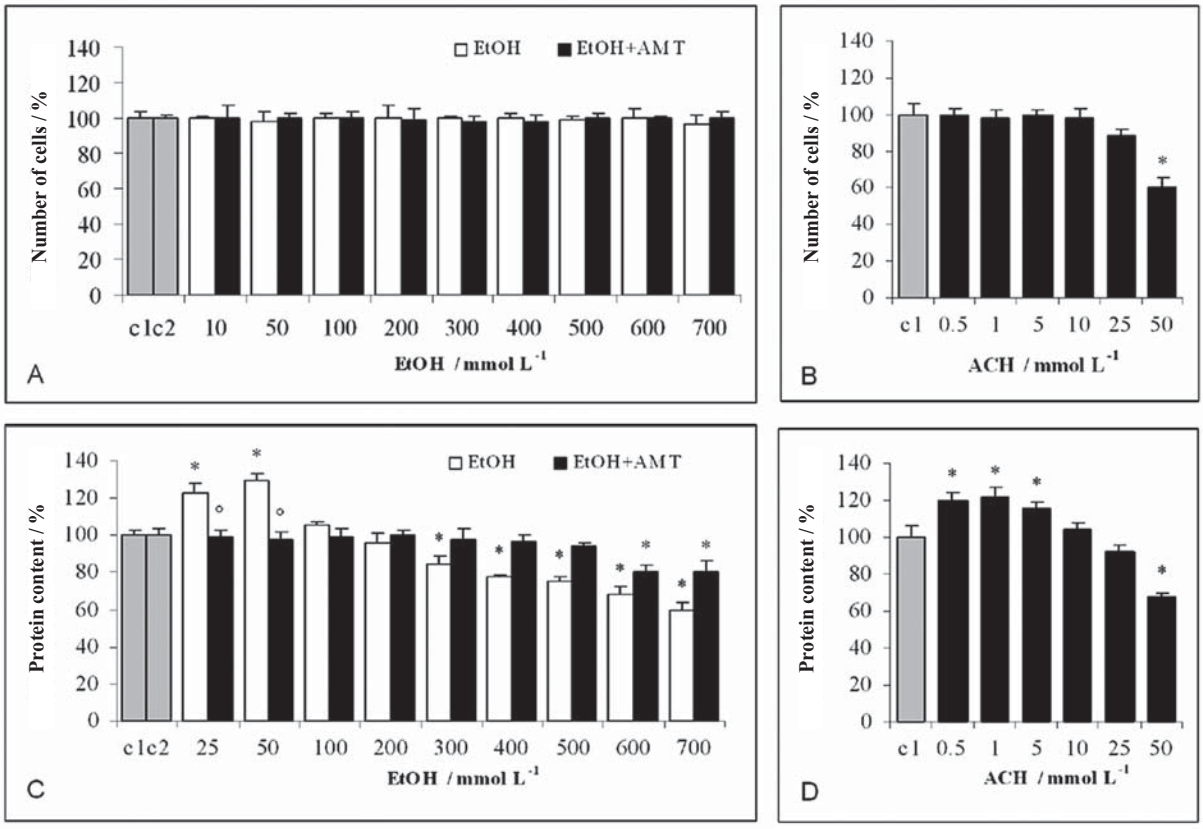

Figure 2 *Effect of ethanol (EtOH) at different concentrations in the presence or absence of catalase inhibitor AMT (10 mmol L^{-1}) on cell count (A) and protein content (C) in cell culture, after 24 hours of incubation. Effect of acetaldehyde (ACH) at different concentrations on cell count (B) and protein content (D) in cell culture, after 24 hours of incubation. Each bar is the mean ± SEM of three independent determinations. *p<0.05 versus control c1; °p<0.05 versus values with the same ethanol concentration in the absence of AMT. c1, control cells - growth medium; c2, control cells, treated with 10 mmol L^{-1} AMT.*

Pre-incubation with AMT reduced ethanol influence on proliferation by 24.1 %, with EC_{50} rising to 436.7 mmol L^{-1} (Figure 3C).

After seven days of exposure, acetaldehyde inhibited cell growth at much lower concentrations than ethanol; the first significant decrease in cell number was observed at 5 mmol L^{-1} acetaldehyde (Figure 3B). EC_{50} was 13 mmol L^{-1}. Protein content first significantly dropped at 5 mmol L^{-1} acetaldehyde, with EC_{50} 8.7 mmol L^{-1} (Figure 3D).

DISCUSSION

Although brain damage associated with chronic consumption of ethanol is multifactorial and depends on a number of ethanol effects on the central nervous system, some data suggest that oxidative metabolism of ethanol and produced substances are involved in the aetiology of these effects (33). Many studies have shown that both ethanol and acetaldehyde disturb astroglial growth and differentiation; however, the mechanism has remained elusive (8, 11, 34-39).

There are reports that ethanol at high concentrations intercalates into cell membranes, increasing membrane fluidity (40). In our study we have shown that ethanol at high concentrations (700 mmol L^{-1} and above) affects cell viability in primary rat cultures. This result is no surprise, because astrocytes are fairly resistant cell species with high antioxidant capacity. Compared to ethanol, acetaldehyde was much more potent; its first significant effect on cell viability was seen at a much lower concentration (50 mmol L^{-1}).

Astrocytes are the most abundant brain cells. As such, they also provide metabolic and trophic support to neurones. Astrocytes are able to respond to an injury with altered phenotype. In such conditions they respond with increased production of intermediate filaments and structural glial fibrillary protein, with cell hypertrophy, and sometimes with cell proliferation

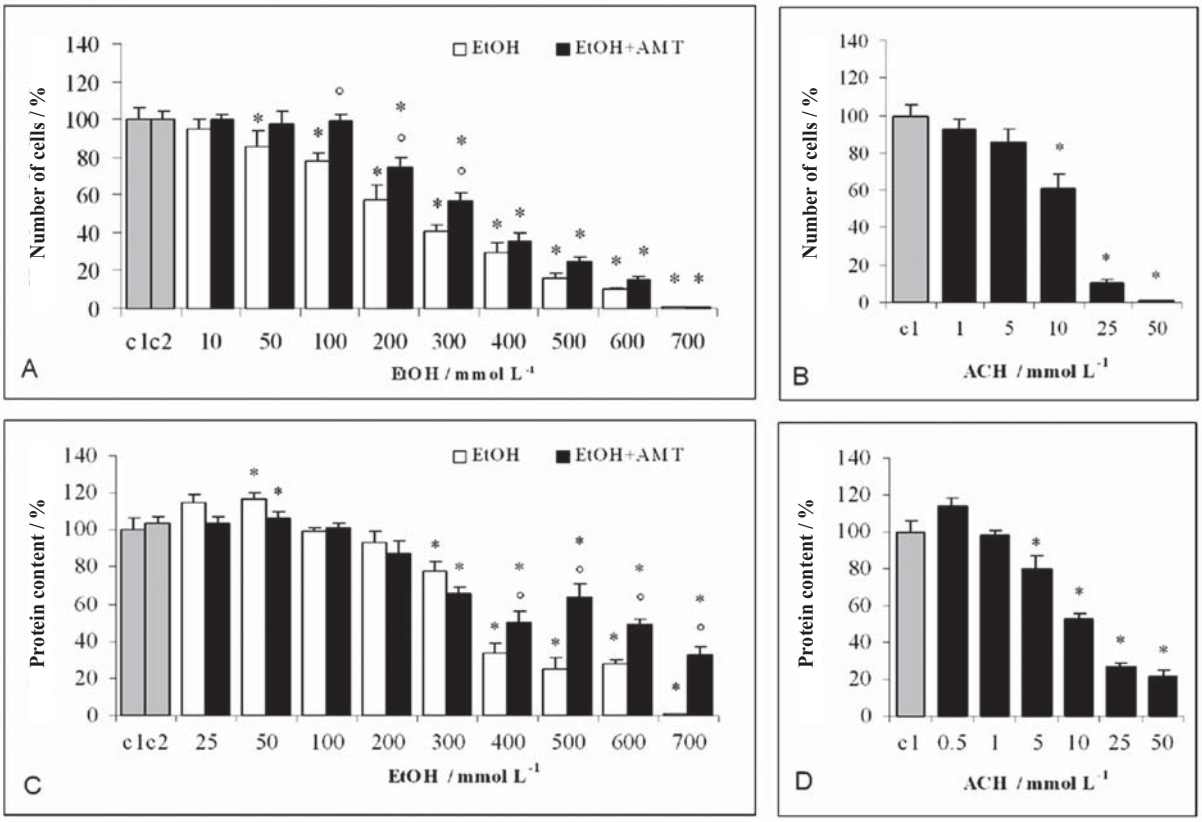

Figure 3 *Effect of ethanol (EtOH) at different concentrations in the presence or absence of catalase inhibitor AMT on cell count (A) and protein content (C) in cell culture after seven days of incubation. Effect of acetaldehyde (ACH) at different concentrations on cell count (B) and protein content (D) in cell culture after seven days of incubation. Each bar is the mean ± SEM of three independent determinations. *p<0.05 versus control c1; °p<0.05 versus values with the same ethanol concentration in absence of AMT. c1, control cells - growth medium; c2, control cells, treated with 10 mmol L^{-1} AMT.*

(41). This could explain the biphasic, hormetic dose-response effect, which we obtained in the cell proliferation test by measuring cell protein content.

We observed the biphasic effect of ethanol after 24 hours and after seven days of exposure; low concentrations of ethanol stimulated protein production, whereas higher concentrations inhibited it.

Astrocytes have a high antioxidant capacity. In oxidative stress, they react with upregulation of a large number of molecules including those controlling the protective system. Therefore, they can protect neurons against toxic damage (42). Watts et al. (43) showed the protective effect of co-cultured astrocytes on neurons, reducing the apoptosis rate after 12-hour and 24-hours exposure to 55 mmol L^{-1} and 88 mmol L^{-1} of ethanol. Neuron protection could be also speculated in our study at ethanol doses of 25 mmol L^{-1} and 50 mmol L^{-1}, with significant increase in protein content after acute exposure. However, protein upregulation was blocked by pretreatment of astrocyte cultures with catalase inhibitor AMT, which decreased acetaldehyde production and, in turn, could have decreased the production of reactive oxygen species (ROS). Decreased ROS level production by neurons co-cultured with astrocytes during the second hour of exposure to 55 mmol L^{-1} and 88 mmol L^{-1} of ethanol was also found by Watts et al. (43).

Both ethanol and acetaldehyde inhibited astrocyte growth after seven days of exposure in a dose-dependent manner, but acetaldehyde was much more toxic. Their inhibitory effect on proliferation was confirmed by low cell count and low protein content in cultures. The first significant decrease in cell count versus control was observed at 100 mmol L^{-1} of ethanol, and the first significant difference in protein content was seen at 300 mmol L^{-1}. This difference could be related to the common astrocyte response to low doses of toxicants by altering the phenotype, which in turn leads to upregulation of a large number of molecules protecting the cell (28). According to basic toxicological data for acute oral toxicity in rats, acetaldehyde is 10 to 20 times a more potent toxin than alcohol (44). Our results for acetaldehyde go along these lines, as we observed the first significant decrease in cell count versus control at 5 mmol L^{-1}.

The mechanism of action of ethanol on astroglial growth is still subject to debate. Proliferation of astrocytes is mainly controlled by soluble mitogenic factors such as growth factors acting on tyrosine kinase-coupled receptors (45, 46). Ethanol has been shown to inhibit basal astroglial proliferation and response to mitogenic factors such as IGF-1 and acetylcholine (35, 47). Ethanol (e.g., 30 mmol L^{-1} to 250 mmol L^{-1}) inhibits cell proliferation (34, 48) and reduces incorporation of [^{3}H] thymidine (49). High concentrations of ethanol alter cell-cycle kinetics of proliferating astrocytes by delaying the passage of the cells trough G1 (49). These effects appear to be dose-dependent. At low concentrations (e.g., 14 mmol L^{-1}) ethanol increases DNA, RNA, and protein synthesis (43). Ethanol also increases astrocyte death, both by necrosis and apoptosis (11).

A number of ethanol effects, including psychopharmacological and neurotoxic effects, are believed to be mediated by its first metabolite, acetaldehyde. However, there are only a few data about the cytotoxicity of acetaldehyde in astrocytes. Acetaldehyde, but not ethanol, increases intracellular calcium level, elevates transglutaminase activity, and causes significant DNA fragmentation and cell nuclei chromatin condensation in astrocytes (50). We have shown that pretreatment with catalase inhibitor AMT significantly diminishes ethanol toxicity (28 % lower after 24 hours of exposure and 24.1 % lower after seven days of exposure). Our results are also in accordance with one of the rare reports about acetaldehyde production in cultured astrocytes, where 10 mmol L^{-1} AMT decreased acetaldehyde metabolism from ethanol by 52 % (51).

To conclude, both ethanol and acetaldehyde show toxic effects on cultured astrocytes and both inhibit proliferation, which depends on the concentration and time of exposure. Long-term exposure of astrocytes to either ethanol or acetaldehyde is more toxic than acute exposure. Acetaldehyde is a much more potent toxin than ethanol. Moreover, at least a part of ethanol toxicity is due to its metabolising to acetaldehyde. Our study may contribute to better understanding of the involvement of ethanol and its oxidative metabolite acetaldehyde in the development of foetal alcohol syndrome due to ethanol exposure during pregnancy and in the development of morphological and functional changes in the CNS in chronic alcoholics.

Acknowledgements

This study was supported by the research grant P3-0067 by the Slovenian Research Agency, Republic of Slovenia.

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Izvleček

PRIMERJAVA TOKSIČNOSTI ETANOLA IN ACETALDEHIDA ZA PODGANJE ASTROCITE V PRIMARNI KULTURI

V študiji smo primerjali toksičnost etanola in njegovega prvega metabolita acetaldehida za podganje astrocite z določitvijo celične viabilnosti in proliferacije. Celične kulture smo tretirali z različnimi koncentracijami etanola, etanola v prisotnosti inhibitorja katalaze 2-amino-1,2,4 triazol-a (AMT) ali z različnimi koncentracijami acetaldehida. Celično viabilnost smo vrednotili s pomočjo testa s tripanskim modrilom, celično proliferacijo pa s štetjem celic in določitvijo koncentracije proteinov po 24-urni, kot tudi 7-dnevni izpostavljenosti.
S študijo smo pokazali, da tako etanol kot tudi acetaldehid v odvisnosti od njune koncentracije zmanjšata celično viabilnost. V študiji proliferacije sta etanol in acetaldehid, v odvisnosti od njunih koncentracij, značilno zmanjšala število celic po 7-dnevni izpostavljenosti. Pri ugotavljanju vsebnosti proteinov smo dobili bifazno krivuljo tako po 24-urni, kot tudi po 7-dnevni izpostavljenosti različnim koncentracijam etanola oziroma acetaldehida. Prisotnost AMT je signifikantno zmanjšala učinek etanola na celično proliferacijo.
Zaključimo lahko, da je dolgotrajna izpostavljenost astrocitov etanolu bolj toksična kot akutna. Acetaldehid je močnejši toksin kot etanol in vsaj del toksičnosti etanola je posledica delovanja njegovega prvega metabolita, acetaldehida.

KLJUČNE BESEDE: *AMT, proliferacija, tripansko modrilo, viabilnost*

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