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Antioxidants do not prevent acrylonitrile-induced toxicity

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Abstract

Several reports have recently described that acrylonitrile (ACN) toxicity resides in its capacity for inducing oxidative stress. ACN can be conjugated with glutathione (GSH), diminishing its cellular content, or being metabolized to cyanide. In the present report, we determine the effect of ACN on the viability of primary-cultured astrocytes as well as the oxidative damage generated by ACN by measuring GSH levels in primary cultured astrocytes. We also analyzed whether the ACN (2.5 mM) toxicity could be avoided by using antioxidants such as taurine (5 mM), *N*-acetylcysteine (20 mM), trolox (100 μM), estradiol (10 μM) and melatonin (100 nM–1 mM). In this cell culture model, antioxidants were not able to prevent ACN-induced cell damage, with the exception of NAC, confirming that only GSH seems to play a key role in ACN-derived toxicity. Additionally, we measured different parameters of oxidative stress such as catalase activity, lipid peroxidation and GSH concentration, as indicators of the potential oxidative stress mediated by the toxicity of ACN, after exposure of Wistar rats to a concentration of 200 ppm ACN for 14 days. At the concentration assayed, we did not find any evidence of oxidative damage in the brain of ACN-treated rats.

Keywords: Acrylonitrile; Oxidative stress; Catalase; Melatonin; Taurine; *N*-acetylcysteine

1. Introduction

Several studies have shown that ACN exposure is associated with the induction of oxidative stress and this has been suggested to have a key role in ACN-induced morphological transformation and carcinogenicity (Zhang et al., 2002). ACN has been described to be mutagenic, teratogenic and carcinogenic in rodents (Kamendulis et al., 1999; Ghanayem et al., 2002) although the mechanisms involved remain poorly understood. ACN generates cyanoethylene oxide (CEO) and cyanide through its metabolism or can directly conjugate with glutathione (GSH), the main intracellular antioxidant (Wang et al., 2002). According to Nerland et al. (2001), both CEO and ACN would react with tissue thiols, leading to a rapid GSH depletion. Because these compounds undergo conjugation with endogenous GSH (Ghanayem et al., 2002), and this conjugation prevents their further metabolism to cyanide, it is likely that GSH tissue levels may influence the eventual metabolism of ACN to cyanide (Chanas et al., 2003). On the other hand, some authors have attributed the carcinogenic effects

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of ACN to CEO, a compound generated by epoxidation of ACN (Guengerich et al., 1981; Recio and Skopek, 1988). CEO hydrolysis via epoxide hydrolase (EH) is considered as the primary route for cyanide formation, which is then in turn converted into thiocyanate via rhodanase and eliminated by urine (Tardif et al., 1987; Chanas et al., 2003). Cyanide is known to uncouple oxidative phosphorylation and it has been described to induce oxidative stress in the brain of acutely treated mice and in cell lines (Johnson et al., 1987; Mills et al., 1996; Kanthasamy et al., 1997). Contrary to early reports, which suggest that GSH depletion is a critical event in the toxicity of ACN (Jiang et al., 1998), its processing to CEO and subsequent transformation into cyanide would be critical in the development of the acute toxicity/mortality of ACN (Wang et al., 2002).

It has been reported that ACN selectively induces oxidative stress in rat brain at the doses that produce carcinogenesis after chronic treatments (Jiang et al., 1998). The brain may be prone to oxidative damage, due to a high pro-oxidant status (Floyd and Carney, 1992) and a lowered antioxidant defence capacity with respect to other organs (Halliwell and Gutteridge, 1999). Glial cell tumours have been reported to begin at about 1 year of ACN exposure in rats (Quast et al., 1980a,b) being astrocytomes the neoplasms of major incidence in these models (Whysner et al., 1998b). In this sense, Mahalakshmi et al. (2003) described the modulation of antioxidant enzymes and DNA fragmentation in brain tissue from rats exposed to 100 ppm of ACN for 14 days. Previously, neural toxicity had been described, after 14 days treatment of 200 ppm of ACN, by Jiang et al. (1998). Therefore, since oxidative stress seems to be the mechanism responsible for the toxicity of the ACN, and consequently in the formation of gliomas, the purpose of this study was to test and analyze the effect of several antioxidants on the ACN-induced toxicity in primary cultured astrocytes, since these cells are more resistant to oxidative stress compared to oligodendrocytes and neurons (Wilson, 1997). In this paper, we studied the effect of taurine (TAU), *N*-acetylcysteine (NAC), trolox (TRX), estradiol (ES) and different concentrations of melatonin (MEL), on the citotoxicity of ACN in primary cultured astrocytes.

2. Materials and methods

2.1. Chemicals

ACN (>99% purity) and melatonin were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Cell culture reagents were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA) except for foetal bovine serum (FBS) that was purchased from Gibco (Invitrogen Life Technologies, Barcelona, Spain). Cell culture flasks and dishes were purchased from Falcon (Becton Dickinson BioScience, Le Pont de Claix, France). All other reagents were obtained from Sigma Chemicals (St. Louis, MO, USA), unless otherwise indicated.

2.2. Primary cell culture and treatment

Astrocytes were obtained from the brain cortex of 1–2 days old Wistar male rats. After being sacrificed by decapitation, brain cortices were separated, fragmented into small pieces and incubated in 10 ml trypsin solution (0.125%, w/v) in phosphate-buffered saline (PBS) at 37 °C for 15 min. Immediately after, trypsin was inactivated by addition of two volumes of complete medium [Dulbecco's modified eagle's medium/Ham's F-12 (DMEM/F12) supplemented with 10% FBS and 1% of an antibiotic-antimycotic mixture (100 U/ml of penicillin, 0.1 mg/ml of streptomycin and 0.25 μg/ml of amphotericin)]. Cells were then recovered by centrifugation for 5 min at 200 × *g* and the pellet obtained was resuspended in Hank's balanced salt solution (HBSS). Cells were isolated by filtering the suspension through 80 and 100 mesh metal grids. After counting, cells were seeded at a density of 3×10^6 cells/ml into poly-lysine-coated flasks in a final volume of 20 ml. Cells were grown at 37 °C an atmosphere of 5% CO_2 and after 7 days, medium was changed to discard neurones. Medium was renewed every 3 days. After 10 days of culture, cells were shaken for 2 h at 100 rpm in order to suspend and discard glial cells other than astrocytes. All the experiments were performed 24 h after, once cells reached 70–80% of confluence. Cell treatments were carried out according to the following procedure: once cells were plated, they were treated for 1 h with/without TRX, TAU, EST, NAC or MEL. After that, they were exposed to a concentration of 2.5 mM ACN for 24 h, after which the corresponding parameters were determined.

Preparation of drugs: *N*-acetyl cysteine (1 M) was freshly dissolved in DMEM prior to be added; trolox and taurine (100 mM) were dissolved in 100% ethanol or in 100% medium culture respectively; estradiol (10 mM) was prepared in 100% ethanol whereas melatonin was freshly prepared as 1 M stock solutions in 50% DMSO/medium culture. Dilutions were carried out in serum-free medium and the control groups received the corresponding concentration of DMSO or ethanol. The exposure of cells to the different antioxidants, without ACN, included in this study they did not induce toxicity on the cells. The concentrations used for each one of the compounds have been chosen according to other reports in which antioxidant capacity in astrocytes and other cell types have been shown (Bastianetto et al., 2000; Dare et al., 2000; Martin et al., 2002a,b; Bajt et al., 2004; Chang et al., 2004).

2.3. Animals and treatments

Twenty-four male Wistar rats (*Rattus norvegicus*) (130.61 ± 8.038 g body weight) were used. Animals were provided by the animal bioterium facility from the University

of Oviedo, and maintained in an environment controlled under constant temperature (25 °C) at a 12-h light:12-h dark cycle (lights on at 07:00). Animals were allowed to access water and food *ad libitum*. Experimental procedures for animal use and care were in accordance to the European Community Council directive (86/609/EEC). Rats were randomly divided into two groups. One served as a control while the other received ACN (200 ppm) dissolved in drinking water according to a method previously described (Jiang et al., 1998). Drinking water, with or without ACN, was changed every 3 days and bottles were weighted everyday for water consumption determination. Body weight was also registered. An estimation of increase of body weight and consumption of drinking water of both groups is plotted and expressed as g/rat/day and ml/rat/day, respectively. After 14 days of treatment, the animals were anesthetized with halothane in oxygen (1 l/min oxygen) and perfused transcardially with ice-cold physiologic saline serum (9‰, w/v NaCl) prior to decapitation. Brains were rapidly excised, frozen and stored at −80 °C, until further use.

2.4. Cell viability assays

In order to determine the effects of several antioxidants on the toxicity induced by ACN, cell viability was measured by determining the activity of lactate dehydrogenase (LDH) released by damaged cells into the medium as described by Decker and Lohmann-Matthes (1988). Cells were plated in 24-well dishes at a density of 1×10^5 cells/ml of complete medium. LDH activity was assayed in both, an aliquot of the culture medium (released LDH) and an aliquot of culture medium after the lysis of cells by incubation for 30 min with 1%, v/v Triton X-100 in PBS (total LDH). Absorbance was measured in an automatic microplate reader (μQuant, Bio-Tek Instruments, Inc, Winooshi, UT, USA) at 490 nm. Results are shown as the percentage of released lactate deshydrogenase *versus* control. We employed the LDH assay for all the antioxidant with the exception NAC, since we observed an interference of this thiol with the technique. Therefore in the case of NAC, we carried out another alternative technique that was tripan blue exclussion assay.

For this purpose, astrocytes were plated in 60 mm dishes at a density of 4×10^5 per plate in 4 ml of complete medium. Once the treatments with NAC and with or without ACN were concluded, cells were trypsinized and collected at $200 \times g$ for 5 min. Cells were stained with Trypan Blue solution (0.4%, w/v) and counted in a Neubauer hemacytometer chamber (Sigma, St. Louis, MO, USA). The number of nonstained and stained cells was counted and percentage of viable cells was calculated. Experiment was performed at least three times and three replicates per plate were counted in each experimental group.

2.5. Glutathione assay

GSH levels were measured as described by Griffith (1980), with minor modifications. For this purpose, astrocytes were cultured in 100-mm plates at a density of 1×10^5 cells/ml. Cells were trypsinized and centrifuged at $200 \times g$ for 5 min. Then, samples were washed and resuspended with 1 ml PBS. For protein determination, 100 μl were used and 900 μl remaining were employed for GSH determination. After collecting cells by centrifugation, they were lysed in 50 μl of a buffer constituted for 5% sulphosalicilic acid and 0.1% Triton X-100 detergent. Solution was shaken and sonicated. The supernatant obtained after centrifugation for 5 min at $200 \times g$ was used for the assay. Each slice of brain tissue was homogenized by using the same buffer above mentioned for astrocytes, and centrifuged at $16{,}000 \times g$ for 30 min. Supernatant was then used for determination of GSH. Each sample was mixed with 0.6 mg/ml of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.248 mg/ml nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was started upon addition of 7.5 μl GSSG reductase (100 U/ml). Absorbance was measured at 412 nm in a Beckman Coulter DU®800 spectrophotometer every 20 s for 2 min. The total concentration of GSH was determined from a standard curve obtained with known amounts of GSH.

2.6. Catalase activity assay

Brain tissue slices were processed and analysed for catalase (E.C.: 1.11.1.6) activity as described by Aebi (1984) with slight modifications by Brown-Borg et al. (1999). Enzyme activity was estimated by using an extinction coefficient of $0.0394\ cm^{-1}\ M^{-1}$.

2.7. Lipid peroxidation assay

Brain tissue slices were homogenized (10%, w/v) in 20 mM Tris–HCl pH 7.4 on ice and centrifuged at $3000 \times g$ for 5 min at 4 °C. Supernatants were then used for evaluation of lipid peroxidation by a colorimetric assay with the *N*-methyl-2-phenylindole test kit (BIOXYTECH® LPO-586 ™ Assay, OXIS International, Inc., Portland, USA), following manufacture's instructions.

2.8. Protein determination

For all the experiments, proteins were quantified in triplicate by the method described by Bradford (1976), using bovine serum albumin (BSA) as standard.

2.9. Statistical analysis

For *in vitro* experiments, all the plotted data resulted from three independent experiments. Results are shown as the mean ± standard error. Statistic analysis was performed with one-way ANOVA followed by a Student–Newman–Keuls test. Statistical significance was accepted when $p < 0.05$.

3. Results

As a brain cellular model, in the present study we used primary-cultured astrocytes. Since there are no available

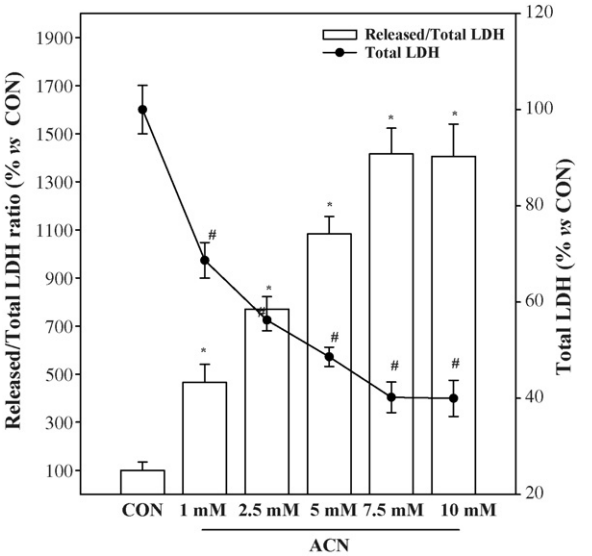

Fig. 1. Effect of dose-response of ACN (1–10 mM) on primary-cultured astrocyte viability for 24 h. Cell viability is expressed as the percentage of the ratio released/total LDH compared to control group. Each value represents the mean ± standard error of the mean ($n = 3$). *$p < 0.05$ *vs.* ratio between released and total LDH of control group. #$p < 0.05$ *vs.* total LDH of control group.

data about the potential toxicity of ACN on this cell type, in order to determine the optimal dose of ACN in primary-cultured astrocytes, a dose-response curve was performed using concentrations of ACN ranging from 1 to 10 mM. Results from these dose-response studies are shown in Fig. 1. Given the results obtained, the dose chosen for the following experiments was 2.5 mM. This concentration was much closed to the LC50 (lethal concentration for 50% of cells), and induced roughly a 40% of cell death (56.25 ± 2.26 total LDH data). Surprisingly, none of the melatonin concentrations used did preserve cell viability, but rather, 10^{-5} M MEL significantly increased the toxicity generated by ACN in astrocytes (11.70 ± 0.02% of released LDH in ACN treated-group

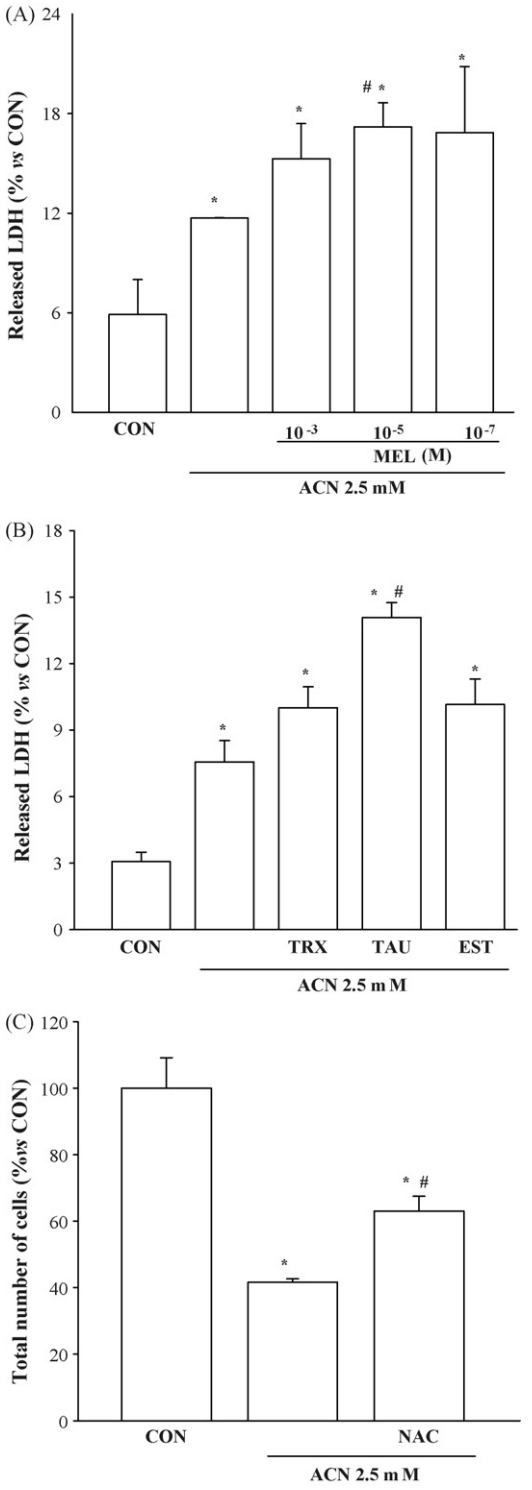

Fig. 2. Cell viability of astrocytes pre-treated for 1 h with/without several antioxidants and treated with/without 2.5 mM acrylonitrile (ACN) for 24 h more. (A) Cell viability of astrocytes treated with with MEL (10^{-3}, 10^{-5} and 10^{-7} M) for 1 h and 2.5 mM ACN. Each value represents the mean ± standard error ($n = 3$). *$p < 0.05$ *vs.* CON, #$p < 0.05$ *vs.* ACN. (B) Cell viability of astrocytes treated with TRX (100 μM), TAU (5 mM) and EST (10 μM) and 2.5 mM ACN. Viability was measured as the percentage of the released lactate deshydrogenase regarding each control. Each value represents the mean ± standard error ($n = 3$). *$p < 0.05$ *vs.* CON, # $p < 0.05$ *vs.* ACN, TRX and EST groups. (C) Total number of cells after treatment with ACN 2.5 mM for 24 h and pre-treated for1 h with NAC (20 mM), determined by Trypan Blue exclusion method. Each value represents the mean ± standard error ($n = 3$). *$p < 0.05$ *vs.* CON, #$p < 0.05$ *vs.* ACN.

versus 17.20 ± 1.44% of released LDH in MEL 10^{-5} M and ACN treated-group) (Fig. 2A). Likewise neither ES (10 μM), nor TAU (5 mM) or TRX (100 μM), all of them well-known antioxidants, recovered or prevented the death induced by the treatment with ACN for 24 h (Fig. 2B). Furthermore, TAU, described in the literature as an antioxidant able to reverse ACN-induced damage *in vivo*, enhanced the toxicity of this drug (7.56 ± 0.97% in ACN treated-group *versus* 14.07 ± 0.67% of released LDH in TAU plus ACN treated-group) (Fig. 2B).

Among the antioxidants included in the study, only NAC (20 mM), a sulfhydryl donor, prevented the decrease of the total number of viable cells in the culture when administered in combination with ACN (41.62 ± 1.03% of ACN treated-cells *versus* 63 ± 4.5% of NAC and ACN treated-cells) (Fig. 2C). Since NAC is considered as a GSH precursor, its effect could be due to GSH conjugation with ACN, decreasing its availability for being metabolized. Thus, we then measured the GSH levels in ACN treated cells and the results are shown in Fig. 3. Treatments of cells for 4 h with ACN partially depleted intracellular GSH whereas 20 mM NAC totally recovered GSH content up to the control levels.

All together results obtained with TAU, TRX, EST and MEL indicate that cellular toxicity induced by ACN cannot be prevented only by antioxidants. Since NAC was the only antioxidant able to prevent ACN toxicity, this prompted us to check whether oxidative stress was the underlying mechanism of ACN action on cell viability. For this purpose, we decided to reproduce a rat model described by Mahalakshmi et al. (2003) and to study different oxidative stress parameters after ACN treatment *in vivo*. As it is shown in Fig. 4, we did not find

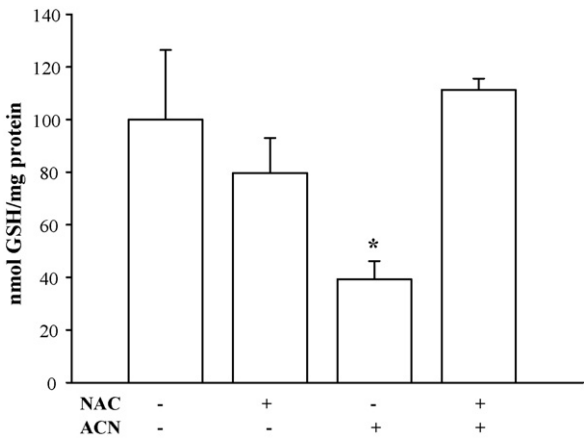

Fig. 3. Glutathione concentration (nmol GSH/mg protein) in astrocytes pre-treated 1 h with 20 mM NAC and treated for 4 h more with 2.5 mM ACN. Each value represents the mean ± standard error ($n = 3$). *$p < 0.05$ *vs.* rest of groups.

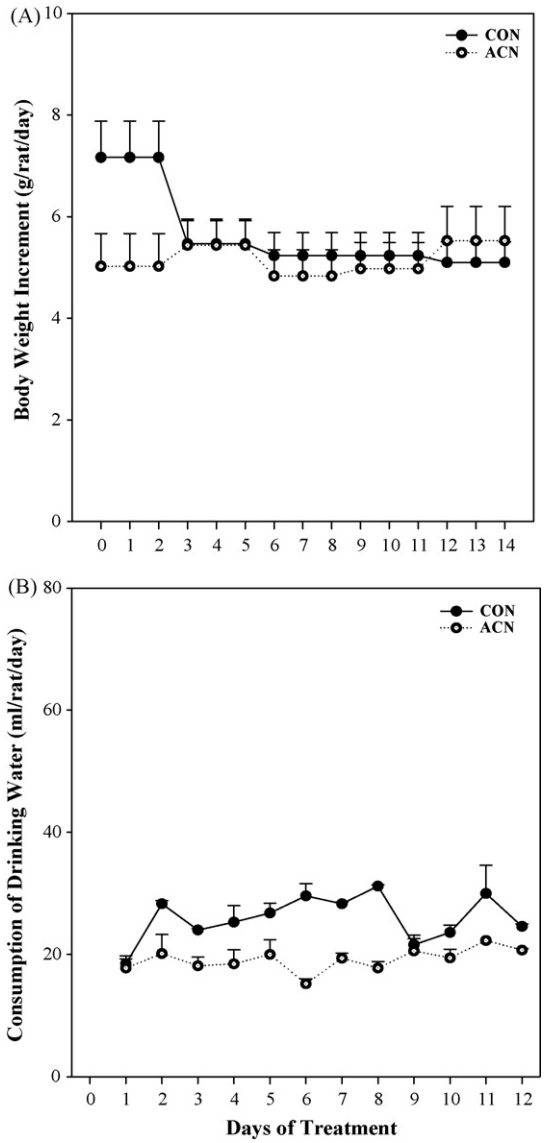

Fig. 4. (A) Estimation of body weight increment of control (black circle) and ACN-treated (dotted circle) rats for the time of the experiment (in days). Values are expressed as g/rat/day. (B) Estimation of consumption of drinking water of animals treated with (dotted circle) or without ACN (black circle) for the time of the experiment (in days). Values are expressed as ml/rat/day.

significant differences neither in body weight increment nor in water consumption between both groups for the time of the study presented here. The effects of ACN in oxidative stress parameters in the rat brain are presented in Table 1. Again, no differences were found in lipid peroxidation products, indicating no increase in oxidative stress in the brain of ACN treated rats. Similarly, we did not observe any significant changes in catalase activity, a known target of ACN and GSH in brain rats

Table 1
Parameters of oxidative stress after ACN treatment. Lipid peroxidation (nmol MDA/mg protein) ($n = 10$), glutathione concentration (nmol GSH/mg protein) ($n = 7$) and catalase activity (U/mg protein) ($n = 10$), were determined in brain tissues of rats exposed for 14 days to ACN (200 ppm). Each value represents the mean ± standard error of the mean (S.E.M.)

	Control	ACN
Lipid peroxidation (nmol MDA/mg prot)	44.39 ± 6.35	39.84 ± 5.44
GSH (nmol/mg prot)	31.96 ± 3.86	32.91 ± 3.77
Catalase activity (U/mg prot)	4.73 ± 0.76	4.45 ± 0.74

(Table 1). Accordingly, reduced and oxidized GSH (GSH and GSSG, respectively) remain also unchanged after ACN treatment.

4. Discussion

In the present work, we report that ACN induces cell death in primary cultured astrocytes. Here we also studied the role of a wide range of concentrations of MEL, the main secretory product of the pineal gland, as well as other well-known antioxidants such as NAC, TAU and TRX as potential agent to prevent ACN-induced toxicity in primary cultures of astrocytes after 24 h treatment with ACN. Antioxidant capacity of MEL, either directly by scavenging free radicals or indirectly by stimulating gene expression of antioxidant enzymes, has been shown by several authors (Kotler et al., 1998; Martin et al., 2002a,b; Mayo et al., 2002; Reiter et al., 2002a,b,c; Tan et al., 2002; Rodriguez et al., 2004; Tomas-Zapico and Coto-Montes, 2005). This would be critical, considering catalase as one of the main targets for ACN, among other antioxidant enzymes. Surprisingly, MEL did not prevent ACN-induced cellular damage. Furthermore, 10 μM MEL significantly increased the toxicity of ACN. Recently, it has been described that MEL activates the electron transport chain, increasing complex I and IV activity (Martin et al., 2002a,b). Since complex IV is the binding site for cyanide to the electron transport chain, and considering that ACN generates cyanide in its metabolism, it would be conceivable that MEL treatment would cause a loop at this level, thus enhancing the toxicity of cyanide. We have recently reported that primary-cultured astrocytes incubated with high concentrations of melatonin neither influenced GSH levels nor the gene expression for different antioxidant enzymes (Martin et al., 2002a,b). Therefore, in this model, its protective role is attributed to its function as a direct free radical scavenger rather than to its indirect actions on gene expression. This hypothesis would also help to explain the data obtained in the present work using pharmacological concentrations of MEL, since ACN toxicity was neither increased nor prevented by treatment with 1 mM MEL, presumably because the main toxicity of this compound resides in the cyanide generation and other mutagenic compounds derived from its metabolism. On the other hand, melatonin concentrations in the nanomolar range require the presence of GSH to be effective (Martin et al., 2002a,b). As we have mentioned previously, one of the major cellular targets of ACN damage is GSH, causing its progressive depletion, which would account for the lack of protective effects in the nanomolar range of the indole. Therefore, the unexpected results obtained with the intermediate dose used might be derived from the absence of both, the free radical scavenging capacity obtained with the higher dose and the ability to increase intracellular GSH levels. Furthermore, at 10 μM, MEL is able to activate complex IV of the electron transport chain which would in turn increase of cyanide toxicity, being this the more plausible theory to explain the unusual effect observed in our study.

We have shown a similar finding with TAU, which has been previously described to rescue ACN-induced damage *in vivo* (Mahalakshmi et al., 2003). TAU regulates an unusual number of biological phenomena, including heart rhythm, contractile function, blood pressure, platelet aggregation, neuronal excitability, cell proliferation or viability and energy metabolism (Schaffer et al., 2000). Martinez et al. (1994) described that sulfhydryl-modifying mercurial reagents increased [3H] TAU efflux under isosmotic conditions and concomitantly decreased the hyposmolarity-evoked efflux. This study demonstrated the essential requirement of sulfhydryl groups for the volume-sensitive taurine efflux. In this way, the most typical biochemical changes caused by ACN are inhibition of sulfhydryl-dependent enzymes and a reduction in GSH concentrations and protein sulfhydryls (EHC 28, 1983). Therefore, the decrease of GSH deposits can induce TAU efflux from cultured astrocytes. Either extracellular elevation or intracellular reduction in TAU induces osmotic imbalances that triggers cell shrinkage (Schaffer et al., 2000), which then increase the concentration of all intracellular constituents and eventually activates phospholipase C, leading to the IP_3 production and Ca^{2+} release from intracellular stores (Hoffmann and Dunham, 1995). In this sense, early studies in apoptosis implicated an increase in cytosolic Ca^{2+} as a direct mediator of DNA fragmentation (McConkey and Nutt, 2001). Thus, we suggest that GSH and energy depletion caused by ACN, will give place to a cascade of osmotic regulation that could end up in cell death.

Among the antioxidants included in the study, only the thiol NAC was able to rescue astrocytes from ACN toxicity. It has been generally assumed that survival-promoting actions of NAC are due to its sulfhydryl donor capacity directly or indirectly, via maintenance of intracellular GSH (Juknat et al., 2005). In our study, we have observed an increase in total number of cells, which implies an attenuation of ACN-induced cell death. Our results confirmed that ACN induce a significant GSH depletion in primary cultured astrocytes. Therefore, the presence of NAC would increase the intracellular pool of GSH, subsequently diminishing the availability of ACN to be metabolized into CEO and cyanide and thus halting the toxicity induced by ACN. On the other hand, NAC could also keep cells alive for more time without affecting the metabolism of ACN.

GSH is found at relatively high concentrations in the brain and is the major route for detoxification of ACN (Kannan et al., 1992). The GSH conjugation and microsomal epoxidation pathways yield distinct metabolic products, which are excreted primarily in the urine (Ahmed et al., 1982). High GSH levels appear to be protective against ACN exposure compatible with animal survival (Whysner et al., 1998a). On the other hand, as it has been described in several reports, the major pathways of ACN are the conjugation with GSH and its metabolism by cytochrome p450 (CYP2E1). Thus, ACN toxicity could be considered as a process that commits different cellular types in the brain. Neurons, particularly in the hippocampus, posses the major constitutive activity of this cytochrome, while astrocytes are the nervous cells with the highest content in GSH, being the origin of the main neoplasms described in rodents after exposure to ACN. It is noteworthy to mention that P4502E1 can generate reactive oxygen species from molecular oxygen and the hyppocampal neurons are very susceptible to oxidative stress (Upadhya et al., 2000).

Given our results in primary cultured astrocytes, we decided to study oxidative stress parameters in a chronic model of ACN treatment. Several authors have suggested ACN as an oxidative stress inducer and this seems to be the mechanism underlying glioma induction after continuous exposure to this compound. However, conflicting results has been obtained regarding to free radicals mediation in ACN toxicity (Kamendulis et al., 1999; Chanas et al., 2003). In this sense, Whysner et al. (1998a) demonstrated that no genotoxic effect of ACN have been found to account for the carcinogenicity of ACN. DNA reactivity of ACN or its metabolites has been detected under certain experimental conditions, but these effects have not been linked to ACN carcinogenesis in the rat target organs. On the other hand, the described induction of oxidative stress by ACN appears to involve the depletion of DNA repair enzymes (Zhang et al., 2000).

In our study, we did not find any change in catalase activity in the group of rats treated with this drug. In this regard, although other reports have found a decreased catalase activity after ACN treatment (Jiang et al., 1998; Mahalakshmi et al., 2003), Kamendulis et al. (1999) have described that this activity shows no significant decrease from the control following 4 or 24 h of ACN exposure in rat astrocytes. In agreement to these studies, we did not observe any changes in the levels of GSH/GSSG in the ACN group. Apparently, this is contrary to a published *in vivo* study in which ACN treatment, at a dose that induces astrocytome in rats, induced an early depletion of GSH in the brain (Jiang et al., 1998). However, other authors showed that ACN treatment did not result in a significant depletion of GSH in rat brain, discarding ROS as mediators involved in the formation of 8-oxodeoxyguanosine (8-OH-dG). Furthermore, the only indicator of damage resulting from ACN exposure in brain DNA described was an increase in the levels of 8-OH-dG in brain DNA from rats exposed to ACN. This lesion is believed to cause HisG46 transversion mutagenesis in the *Salmonella* reversion assay (Cheng et al., 1992). However, this mechanism of DNA damage would presumably be epigenetic, involving reactive oxygen species rather that involving direct adduct formation by ACN or its metabolites (Whysner et al., 1998a). Authors concluded that 8-OH-dG formation did not appear to involve lipid peroxidation or disruption of antioxidant defences. This affirmation would agree with our results, since levels of lipid peroxidation among the groups studied were unchanged. Other studies carried out by different groups support our results, since they did not observe lipid peroxidation after ACN treatment in *in vitro* studies, at any concentration or time point (Kamendulis et al., 1999).

The discrepancy of our results respect to other *in vivo* studies employing ACN could be due to either a difference in the assay method performed, the strain of rats employed for the study or the treatment of the samples. Indeed the first antioxidant defence is the blood, and thus the absence of this in our perfused samples, would give place to the exclusive determination of oxidative parameters in brain. In addition, these variations could also depend on the rat strain, since some metabolites of ACN such as cyanoethanol and cyanoacetic acid have been found in the urine of Wistar rats exposed to ACN (Lambotte-Vandepaer et al., 1981) but not in F344 rats or B6C3F1 mice (Kedderis et al., 1993), indicating differences in the sensitive of ACN.

The present results indicate that the main pathway by which ACN cause cell damage in astrocytes can-

not be halted by the employment of antioxidants. Both, ACN and CEO bind avidly to protein sulfhydryls, and CEO is also mutagenic and likely to be responsible for the cellular damage and carcinogenicity induced by ACN.

5. Conclusion

Here, we conclude that although ACN has been reported to generate oxidative stress and this is considered, according to the literature, as a potential mechanism involved in the development of nervous system tumours in rodents, this seems not to be the exclusive mechanism underlying the ACN toxicity. Furthermore, antioxidants alone are not adequate as protectors against ACN toxicity *in vitro*. We assume that this is due to the complexity of its metabolism, the toxicity and mutagenicity of the different compounds derived from ACN, as well as its multiple cellular targets.

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