ELSEVIER

Neurochemistry International 42 (2003) 385–391

NEUROCHEMISTRY
International

www.elsevier.com/locate/neuint

WR-2721 (amifostine, ethyol®) prevents motor and morphological changes induced by neonatal X-irradiation

L.R. Guelman [a,b,*], M.A. Zorrilla Zubilete [a,b], H. Rios [c], L.M. Zieher [a,b]

[a] *1ª Cátedra de Farmacología, Facultad de Medicina, Universidad de Buenos Aires (UBA), Paraguay 2155—piso 15° (1121), Buenos Aires, Argentina*

[b] *Instituto de Neurociencias Aplicadas, Hospital Escuela "José de San Martín", Facultad de Medicina, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina*

[c] *Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*

Received 15 May 2002; received in revised form 16 August 2002; accepted 23 August 2002

Abstract

Neonatal X-irradiation induces permanent abnormalities in cerebellar cortex cytoarchitecture and neurochemistry, as well as impairment in motor gait. The aim of the present work was to examine the potential protective properties of WR-2721 (Amifostine, Ethyol®), a free radical scavenger, against the above mentioned alterations by using a previously described neuroprotection assessment protocol. Pre-irradiation treatment with amifostine was effective in partially preventing the cerebellar morphological damage and the motor gait impairment induced by ionizing radiation. No changes in cerebellar noradrenaline (NA) levels were detected in amifostine-treated irradiated animals. These results suggest that it is possible to counteract radiation-induced damage in the cerebella and motor gait of neonatal rats through oxygen free radical scavenger administration prior to irradiation. The presence of the agent *before* the injury occurs, favors the efficacy of amifostine neuroprotective activity. Clinical implications of this model are related to the daily exposure of many people to different sources of radiation (accidental, diagnostical or therapeutical).

Keywords: WR-2721; X-irradiation; Neuroprotection

The exposure of mammals to ionizing radiation during the neonatal period produces marked alterations in a variety of target tissues. The cytoarchitecture and functioning of different structures of the developing central nervous system (CNS) have shown to be affected by X-rays (Schull et al., 1990; Altman et al., 1967; Altman and Anderson, 1971; Ratan et al., 1994; Inouye and Kameyama, 1983; Kameyama and Inouye, 1994).

Previous findings of our laboratory showed that neonatal X-irradiation induces permanent abnormalities in cerebellar cortex cytoarchitecture (granule cell loss and disarrangement of Purkinje cell monolayer) and neurochemistry (increased levels of noradrenaline (NA) in the cerebellum) as well as an impairment in motor gait (ataxic gait) (Dopico et al., 1989, 1990a,b, 1991; Guelman et al., 1993, 1996; Dopico and Zieher, 1993). These results have demonstrated that this neonatal model is highly radiosensitive and that it is a useful tool to test potential neuroprotective substances to prevent radiation-induced abnormalities. (Guelman et al., 2000, 2001).

The involvement of reactive oxygen species (ROS) in neurodegenerative diseases as well as in various CNS injuries, such as ionizing radiations, has been documented in the last few years (Kermer et al., 1999; Michikawa et al., 1994; Halliwell, 1992; Duchen, 2000).

The brain consumes more than 20% of the total incoming oxygen, therefore it is one of the most active organs of the body. For this reason, ROS are generated at greater levels in comparison with other tissues. In addition, the antioxidant status of brain membranes is lower than other tissues, leading to an unsuccessful elimination of free radicals with the consequent onset of oxidative stress.

Radiation damage in biological systems is initiated by ROS during the energy deposition events. Several mechanisms would contribute to the subsequent radiation damage over a wide time scale: ROS induce damage within a few microseconds from their formation, while gross pathological lesions may arise from biochemically altered

* Corresponding author. Tel.: +54-11-4508-3733; fax: +54-11-4508-3733.
E-mail address: lguelman@fmed.uba.ar (L.R. Guelman).

0197-0186/02/$ – see front matter
PII: S0197-0186(02)00136-5

macromolecules after a much longer period, with a high probability to develop cancer (Singh et al., 1990).

Therefore, substances capable of decreasing the production or to scavenging the excess of ROS have gained interest in recent years because of their potential use as neuroprotective agents, demonstrating that ROS-mediated damage might be prevented in different experimental models (Maxwell, 1995; Richardson, 1999; Held, 1985). Scavenging antioxidants react preferentially with free radicals and prevent vital structures from free radical attack, oxidizing them to products with insufficient reactivity.

WR-2721 (amifostine or ethyol®) is a thiophosphate prodrug that is de-phosphorylated in vivo to the radioprotective thiol, *N*-2-mercaptoethyl-1,3-diamino propane (MDP or WR-1065) and is thought to have scavenging properties (Santini and Giles, 1999; McDonough et al., 1992; Tabachnik Schor, 1987). Upon entering into the tissues, it is oxidized to a disulfide by donating hydrogen ions and reducing oxidant species. The in vivo peak tissue concentration of WR-2721 is achieved 10–30 min after injection, suggesting that it should be present at the time of the injury, or should be administered immediately after the neurotoxic insult.

WR-2721 was initially developed during the cold war by the Walter Reed Army Institute as a radioprotectant because of its ability to scavenge free radicals generated after radiation exposure in case of a nuclear attack. Later, this drug was studied for a potential role in protecting normal tissues from the harmful effects of therapeutic radiation and chemotherapy in oncologic patients, although few neuropharmacological intervention strategies are currently available for the treatment of brain disorders that involve free radical injury. Therefore, a free radical scavenger such as WR-2721 would be a potentially effective protector in ROS-mediated radiation injury.

The very few existing reports about radioprotection with WR-2721 to the nervous system have shown negative results (McDonough et al., 1992; Alaoui et al., 1995), mainly due to its inability to cross the blood-brain-barrier in adult brain. The present neonatal model offers an advantage compared to other animal models studied, as the high blood-brain barrier permeability enables an easy access of different agents to CNS.

The aim of the present work was to examine the potential protective properties of WR-2721 on the alterations in motor gait and cerebellar cortex cytoarchitecture and neurochemistry induced by neonatal X-irradiation, using the previously described neuroprotection assessment protocol (Guelman et al., 2001).

Present data demonstrate, at both cytoarchitectural and motor levels, a long-term radioprotection by WR-2721, a free radical scavenger, when administered *prior* neonatal exposure to X-irradiation and therefore suggests that ROS damage might be in part responsible for radiation injury in this neonatal model.

1. Experimental procedures

1.1. Materials

WR-2721 was a generous gift of Schering-Plough Argentina. All other reagents were obtained from Sigma, St. Louis, MO.

1.2. Animals

Newborn littermate Wistar rats of both sexes (10 pups per litter) were maintained with their dams up to weaning and then given rat chow and water ad libitum. The light–dark cycle was of 12 h and the room temperature was 25 °C. Animals were separated into different experimental groups, following random mixing across multiple dams: (1) control, (2) control + WR-2721, (3) X-irradiated, and (4) X-irradiated + WR-2721.

1.3. Pharmacological treatment

100 μg of WR-2721 per gram of body weight were dissolved in 100 μl of saline and administered subcutaneously (SC) to neonatal pups from groups 2 and 4, 30 min prior exposure to sham or X-irradiation. Pups from groups 1 and 3 were similarly injected with equal volumes of isotonic saline.

1.4. Irradiation procedure

The bodies of rat pups from groups 3 and 4 were protected with a 4 mm thick lead shield, and the cephalic ends were exposed to X-rays up to 72 h of postnatal (PN) life. The radiation source was a 220 kV Phillips X-rays unit for deep radiotherapy (Phillips Groeilmpen Fabrieken, Eindhoven, Netherlands), operating at 8 mA. The distance to the target was fixed at 50 cm, and the beam was filtered using 0.5 mm Cu^{2+} and Al^{3+} filters. The effective energy (EE) was 84 kV, the dose rate was 1 Gy per min and the total single dose was 5 Gy. Dosage levels were monitored using a Simplex dosimeter (Simplex Universal Dosimeter Physikalisch Technische Werkstatten, Freiburg, Germany). Rats from groups 1 and 2 were handled similarly to their respective littermates from groups 3 and 4, but were not exposed to X-rays (sham irradiation).

1.5. Neuroprotection assessment

It was performed according to Guelman et al. (2001). The following sections describe the different parameters evaluated in control, irradiated and irradiated-treated animals.

1.5.1. Gait evaluation

Gait of rats from the experimental groups described above was evaluated at PN day 30 using an ad hoc quantitative test (Guelman et al., 1993). Briefly, rats were allowed to

walk freely on a non-moving, paper-covered, flat surface, having their forelimbs and hindlimbs painted with two different coloured inks. Both the distance between the centers of hindlimb footprints (HH) and the distance between the centers of the forelimb footprint and the ipsilateral hindlimb footprint (HF), taken from successive steps, were measured.

1.5.2. Noradrenaline determination

Animals were killed by decapitation at PN day 30 or 90, and their cerebella were dissected out. Cerebellar tissue was homogenized in 0.4 N perchloric acid solution and kept at 4 °C for 24 h. The homogenate was centrifuged at 3500 rpm for 10 min. Noradrenaline was isolated by adsorption to alumina columns and its concentration determined fluorometrically, as previously described (Adler-Graschinsky et al., 1972; Laverty and Taylor, 1968).

1.5.3. Histological procedure

1.5.3.1. Calbindin D28K immunohistochemistry. Perfusion fixation was performed according to a modification of the original method (González Aguilar and De Robertis, 1963). The rats were perfused with a solution containing 4% paraformaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.3–7.5. Dissection of cerebella was followed by immersion in the same fixative solution for additional 6–8 h. The fixed cerebella were dehydrated in ethanol, transferred to 1:1 (v/v) ethanol–toluene, cleared in toluene and embedded in Paraplast. Ribbons were cut at 15 μm sections and mounted on glass slides previously dipped in 0.5% (w/v) gelatin and air dried. Microwave heating (5 min at 750 W) in distilled water was used to enhance the binding of the primary antibody to the tissue (Evers and Uylings, 1994).

The immunostaining was performed using a modified procedure of peroxidase anti-peroxidase (PAP) Sternberger technique. Slices were incubated with the primary antibody (Khan, 1993; Tandler et al., 1997) at a dilution of 1:400 in 1% normal sheep serum and 0.1% Triton X-100 (TS solution) for 24–48 h at 20–24 °C. Afterwards, the slices were washed in TS for 20 min and immersed in the secondary antibody (GAM, Sigma, 1:100) for 3 h. After rinsing again in TS (3 × 10 min), sections were incubated in PAP–mouse complex (Sigma, 1:100) for 3 h.

After washing the sections again in TS (3 × 10 min) and in Tris–HCl 0.05 M, pH 7.3–7.5, immunoreactivity was detected by the peroxidase-DAB reaction, at a concentration of 40 mg% (w/v) for 5 min. The reaction was stopped by immersing the slides first in tap water and then in distilled water. Finally, sections were passed to absolute ethanol and xylene and mounted in Canada Balsam.

1.5.3.2. Nissl staining. The Paraplast was removed with xylene and passed through graded ethanol to distilled water. Sections were stained with 0.2% toluidine blue in 0.06 M potassium biphthalate (pH 4.0–4.2) for 10 min. After rinsing in distilled water, sections were immersed in 5% (w/v) ammonium molybdate for 5 min, dehydrated rapidly in ethanol, passed to xylene and mounted in Canada balsam.

1.5.3.3. Microphotography. Nissl stained sections and calbindin immunostained sections were observed and photographed with a Zeiss Axiophot light microscope.

Histological fields were randomly chosen.

1.6. Statistical analysis

Data were analyzed using ANOVA. The post-hoc analysis to test the significance of the difference between individual means, was performed according to Bonferroni (Hochberg and Tamhane, 1987). Data are expressed as means of 3–5 experiments ± SEM, each with three animals per treatment group.

2. Results

2.1. Motor gait evaluation in amifostine-treated, irradiated rats

Figs. 1 and 2 show that the motor gait of 30 days old rats was significantly impaired after neonatal radiation exposure when compared to matched control animals (black and open bars, for control and irradiated rats, respectively) either in HF ($P < 0.01$) or in HH ($P < 0.01$) parameters. When amifostine was administered 30 min prior to irradiation, HF and HH values were improved, resulting significantly different from irradiated, non treated values (Fig. 1, HF: $P < 0.05$; Fig. 2, HH: $P < 0.01$). The neuroprotective indexes (irradiated, treated/irradiated, non-treated) were 0.84 ± 0.012 and 0.92 ± 0.035 for HF and HH, respectively.

2.2. Cerebellar morphological changes in amifostine-treated, irradiated rats

Fig. 3 shows that cerebellar cytoarchitecture was disrupted in 30 days old rats irradiated at birth (see Figs. 3A and B for control and irradiated cerebella, respectively); the thickness of cerebellar granule cell layer was reduced, and Purkinje cells—normally arranged in a monolayer—were scattered within the cortex.

Amifostine pre-treatment was effective in partially preventing granule cell loss and PK cells disarrangement (Figs. 3C).

2.3. Cerebellar noradrenaline concentration evaluation in amifostine-treated, irradiated rats

Finally, cerebellar NA concentration was increased in 30 days-old rats X-irradiated at birth. However, this increase was not prevented in amifostine-treated X-irradiated animals when compared to vehicle treated irradiated rats (Table 1), even when it was evaluated at 90 days PN.

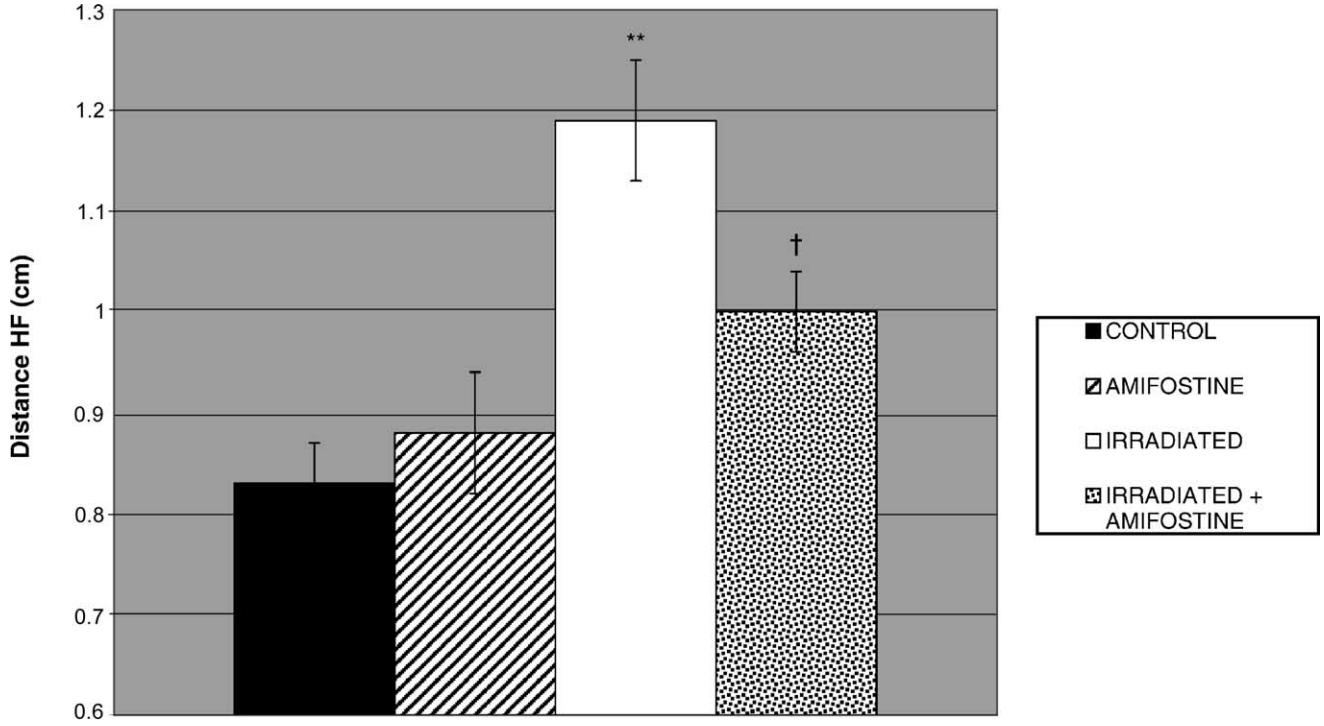

Fig. 1. Effect of amifostine pre-treatment on the gait of 30 days old rats X-irradiated within 72 h after birth. HF: distance between ipsilateral hind and forelimb footprints. Values are expressed as mean ± SEM ($n = 6$–7 experiments, each with three rats per treatment group). **, $P < 0.01$, when compared to controls; †, $P < 0.05$, when compared to irradiated, not amifostine-treated animals. HF values in X-irradiated animals are significantly different from control rats ($P < 0.01$). In amifostine-treated, X-irradiated animals, values are significantly different from those from irradiated rats ($P < 0.05$) and not significantly different from those of control animals.

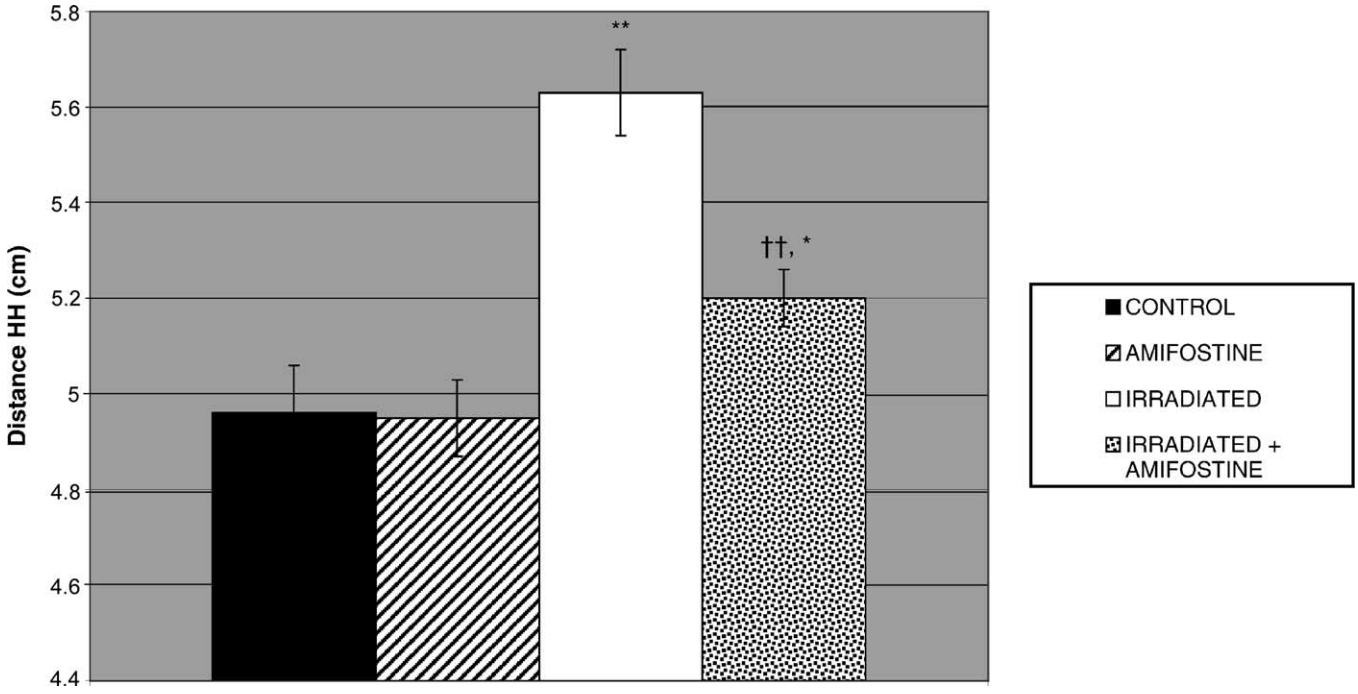

Fig. 2. Effect of amifostine pre-treatment on the gait of 30 days old rats X-irradiated within 72 h after birth. HH: distance between hindlimb footprints. Values are expressed as mean ± SEM ($n = 6$–7 experiments, each with three rats per treatment group). *, **, $P < 0.05$ and 0.01, respectively, when compared to controls; ††, $P < 0.01$, compared to irradiated, not amifostine-treated animals. HH values in X-irradiated animals are significantly different from control rats ($P < 0.01$). In amifostine-treated, X-irradiated animals, values are significantly different from those from irradiated rats ($P < 0.01$) and significantly different from those of control animals ($P < 0.05$).

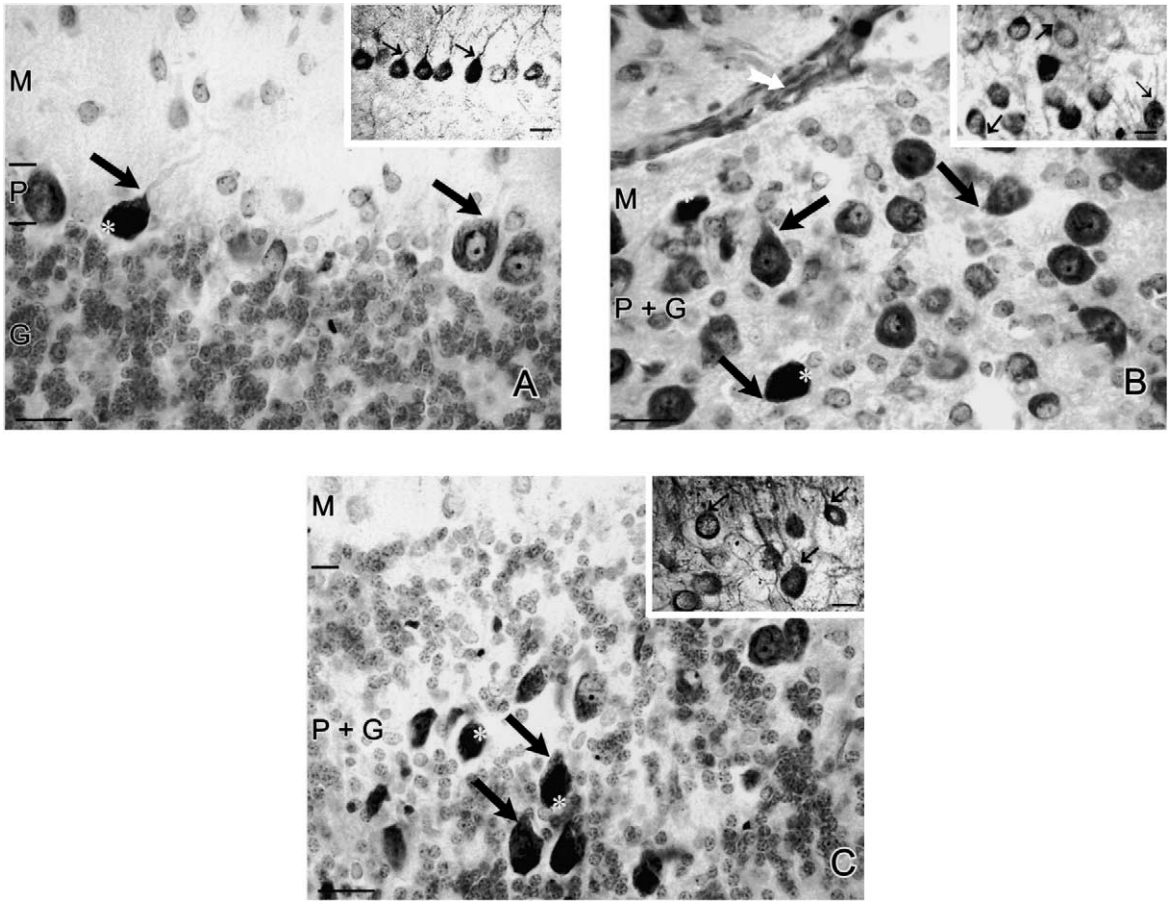

Fig. 3. Effect of amifostine pre-treatment on the cerebellar cortex cytoarchitecture of 30 days old rats following exposure to neonatal X-irradiation. Pictures show light micrographs of parasagittal sections (10 μm thick) through the cerebellar hemisphere. Calibration bar: 20 μm. Inserts, in the three microphotographs, show a Calbindin immunostaining of the cerebellar cortex. (A) In controls, three layers can be clearly distinguished: molecular (**M**), Purkinje layer (**P**) and granular layer (**G**). All Purkinje cells have their primary dendrite oriented towards the pial surface (arrows). Intensity of Nissl staining appeared not uniform among individual Purkinje neurons, as previously described by other authors (Tandler et al., 1997; Khan, 1993). In "dark" Purkinje neurons the nucleus is difficult to see owing to the intense staining of the perikarya (asterisk). The second subpopulation of Purkinje neurons "light" appeared rounded and larger, the nucleus and nucleolus can be observed. (B) In X-irradiated cerebellum, a complete cytoarchitectural disorganization and shrinkage of the cerebellar cortex was observed. Purkinje cells are scattered within the cortex, showing a definite absence of the normal monolayer arrangement. Moreover, molecular, Purkinje and granular layer coexist in an unique layer (M+P+G). Purkinje neurons have their primary dendrite randomly oriented (arrows). Dark and light Purkinje neurons can be observed as in control animals. The white arrow marks the pial surface. (C) In X-irradiated rats treated with amifostine prior to irradiation, some reduction in the thickness of molecular layer can be observed, and Purkinje and granular neurons share a common layer. The general cytoarchitecture of the cortex is preserved and the majority of Purkinje cells are partially arranged in a monolayer, but some are scattered within granular layer. Most of Purkinje cells have their primary dendrite oriented towards the molecular layer (arrows); this feature is clearly evident when Purkinje neurons are stained with anti-Calbindin (insert).

3. Discussion

Neuroprotective activity of WR-2721 was evidenced in an in vivo model of neonatal cephalic X-rays exposure, in which radiation-induced morphological and motor abnormalities were partially prevented. The action of this agent would be mainly related to its ability to limit the damage due to an overproduction of free radicals after radiation exposure (Santini and Giles, 1999). Several studies have shown that radiation-induced cell death follows the apoptotic pathway; this process might be triggered by ROS and can also might induce their generation (Bolaris et al., 2001).

In the present work, a ROS scavenger has been effective in preventing motor gait impairment and cerebellar cortex cytoarchitecture alterations, suggesting that the observed abnormalities could be in part induced by ROS damage produced as a consequence of radiation exposure. In addition, the previously found temporal correlation between motor and morphological alterations (Guelman et al., 1993) and the observed improvement of both parameters after amifostine administration, support the hypothesis that ROS primarily affect cerebellar cells, leading irreversibly to motor gait impairment and suggesting that the radiation-induced abnormalities in brain morphology could underlie the behavioral alterations seen in adult animals neonatally irradiated.

Table 1
Effect of amifostine pre-treatment on cerebellar NA concentration of 30 days old rats X-irradiated at birth

Treatment	NA concentration (μg/g wet tissue)
Control	0.31 ± 0.01
Amifostine	0.41 ± 0.03
Irradiated	0.70 ± 0.09**
Irradiated + amifostine	0.65 ± 0.04**

Values are expressed as μg of noradrenaline per gram of wet cerebellar tissue and are expressed as mean ± SEM (n = 6–7 experiments, each with three rats per treatment group). Cerebellar NA levels in X-irradiated animals are significantly different from control rats levels ($P < 0.01$). In amifostine-treated, X-irradiated animals, values did not differ significantly from those from irradiated rats and remain significantly different from those of control animals ($P < 0.01$).

** Significantly different ($P < 0.01$) from control cerebella.

Radiation toxicity may be divided in early (acute) and late (chronic) reactions. The first involves ROS generation and the second involves neuroplastic mechanisms. Neuroprotective agents are able to restore or prevent these long-term alterations derived from brain damage by modifying a variety of pathways that are involved in the different mechanisms of initial damage: Ca^{2+} increase, apoptosis, energy expenditure impairment, excitotoxicity, mitochondrial impairment, neurotrophic factors signaling, CREB regulation, Bcl-2 expression and ROS-mediated damage (Alaoui et al., 1995; Ciani et al., 1996; Kermer et al., 1999) that could finally lead to cell death. Clinical trials have demonstrated that amifostine can reduce both acute and late radiation-induced toxicities (Weiss and Landauer, 2000; Curran, 1998).

These data are consistent with previous results obtained with another agent, GM1 (Guelman et al., 2000), which was found also to partially prevent the onset of the radiation induced morphological and motor alterations. The fact that two agents acting through different mechanisms have been effective in the prevention of the same radiation-induced alterations, suggests that a multiplicity of converging pathways are triggered by ionizing radiation. The difference between the time of effective administration of the agents (pre or post-irradiation) might be attributed to their different mechanisms of action, amifostine mainly acts on the initial stages of damage, whereas GM1 helps to repair ROS-induced membrane damage.

The lack of effect of either amifostine or GM1 on the altered cerebellar NA levels observed in irradiated animals indicate that both agents might probably protect cerebellar cells (with a consequent improvement of morphological and motor abnormalities) without modifying input afferent pathways from brainstem noradrenergic nuclei (e.g. NA innervation).

The present study is amongst the few reports in the literature in which different CNS areas are in vivo protected by amifostine (McDonough et al., 1992; Tabachnik Schor, 1987). Results reported by Alaoui et al. (1995), who failed to find restoration in the hippocampus of rats treated with amifostine *after* irradiation, support the need of a pre-irradiation treatment. Since free radicals are produced early after radiation injury occurs, a major problem emerges on the delivery of enough neuroprotective agent in the short time available. The protection against radiation lethality in mice (Landauer et al., 1988) and normal peripheral autonomic neurons in the chemoterapeutical treatment of neural crest tumors (Tabachnik Schor, 1987) reported by other authors after a *pre*-treatment with amifostine, was supported by the present results, suggesting that amifostine should be administered shortly *before* the insult in order to be protective.

The present study, as well as previous findings (Guelman et al., 2000) showing *partial* (and no total) prevention and/or restoration of motor and morphological alterations, suggests that other mechanisms besides ROS-mediated cell damage could contribute to radiation-induced injury in this model. Therefore, it might be possible that a combination of agents interfering with different pathways of the mechanism of action of the radiation, could increase the degree of protection against radiation injury, synergizing their action and leading to a greater protection (e.g. combination of the following agents: glutamatergic antagonists, GM1 ganglioside, calcium chelators, antiapoptotic agents, free radical scavengers, etc.). In consequence, a multipharmacological approach could be used in different types of neural insults.

This model of neuroprotection could be extended to other disorders in which CNS vulnerability is increased or when neuroplasticity mechanisms—prioritarie for motor, cognitive and affective CNS processing—are impaired (Manji et al., 2001). Therefore, the present model could be useful to assess in vivo neuroprotection by amifostine, and the drug could be useful to treat other CNS injuries, such as ischaemia, anoxia, etc.

In conclusion, the neonatal radiation model is highly susceptible to be pharmacologically modified and have clinical implications, since many people are daily exposed to different sources of radiation (accidental, diagnostical or therapeutical). These results have proved that amifostine is able to counteract radiation-induced damage in neonatal rats through its ROS scavenging activity, supporting the use of radical scavengers in this and other brain injuries that involve ROS generation.

Acknowledgements

We thank Dr. O. Furia and Dr. D. Feld from the Oncology City Hospital, Buenos Aires, Argentina, for assistance with irradiation of the animals, Dr. J.J. López for the helpful discussion and A. Karpluk from the Scientific and Technologic Research National Council (CONICET), Buenos Aires, Argentina, for his skillful technical assistance. This work was supported by grants from the University of Buenos Aires (ME 077) and CONICET (PIP724) to LMZ.

References

Adler-Graschinsky, E., Langer, S., Rubio, M., 1972. Metabolism of norepinephrine released by phenoxybenzamine in isolated guinea-pig atria. J. Pharmacol. Exp. Ther. 180, 286–301.

Alaoui, F., Pratt, J., Trocherie, S., Court, L., Stutzmann, J.M., 1995. Acute effects of irradiation on the rat brain: protection by glutamate blockade. Eur. J. Pharmacol. 276, 55–60.

Altman, J., Anderson, W.J., 1971. Irradiation of the cerebellum in infant rats with low-level X-ray: histological and cytological effects during infancy and adulthood. Exp. Neurol. 30, 492–509.

Altman, J., Anderson, W.J., Wright, K.A., 1967. Selective destruction of precursors of microneurons of the cerebellar cortex with fractionated low-dose X-rays. Exp. Neurol. 17, 481–497.

Bolaris, S., Bozas, E., Benekou, H., Philippidis, H., Stylianopoulou, F., 2001. In utero radiation-induced apoptosis and *p53* gene expression in the developing rat brain. Int. J. Radiat. Biol. 77 (1), 71–81.

Ciani, E., Voltattorni, M., Rolseth, V., Contestabile, A., Paulsen, R.E., 1996. Inhibition of free radical production or free radical scavenging protects from the excitotoxic cell death mediated by glutamate in cultures of cerebellar granule neurons. Brain Res. 728, 1–6.

Curran, W.J., 1998. Radiation-induced toxicities: the role of radioprotectants. Sem. Radiat. Oncol. 8 (4 suppl 1), 2–4.

Dopico, A.M., Zieher, L.M., 1993. Neurochemical characterization of the alterations in the noradrenergic afferents to the cerebellum of adult rats exposed to X-irradiation at birth. J. Neurochem. 61, 481–489.

Dopico, A.M., Mayo, J., Zieher, L.M., 1990a. Regional changes in central noradrenaline levels after X-irradiation at birth. Acta Physiol. Pharmacol. et Ther. Latinoam. 40, 45–55.

Dopico, A.M., Rios, H., Mayo, J., Zieher, L.M., 1990b. Increased activity of tyrosine hydroxilase in the cerebellum of the X-irradiated dystonic rat. Mol. Chem. Neuropathol. 13, 129–143.

Dopico, A.M., Zieher, L.M., Rios, H., Mayo, J., 1991. Critical postnatal period for the induction of a long-term increase in the noradrenaline content of rat cerebellum by X-irradiation. Neurochem. Int. 19 (1/2), 61–69.

Dopico, A.M., Zieher, L.M., Mayo, J., Altschuller, N., Lopez, J.J., Jaim-Etcheverry, G., 1989. Long-term changes in noradrenaline (NA) and dopamine (DA) contents of rat cerebellum following neonatal X-irradiation. Neurochem. Int. 15 (1), 97–105.

Duchen, M.R., 2000. Mitochondria and calcium: from cell signalling to cell death. J. Physiol. 529 (1), 57–68.

Evers, P., Uylings, H.B.M., 1994. Effects of microwave pre-treatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. J. Neurosci. Methods 55, 163–172.

González Aguilar, F., De Robertis, E., 1963. A formalin-perfusion method for histophysiological study of the central nervous system with the electron microscope. Neurology (Minneap.) 13, 758–771.

Guelman, L.R., Zieher, L.M., Rios, H., Mayo, J., Dopico, A.M., 1993. Motor abnormalities and changes in the noradrenaline content and the cytoarchitecture of developing cerebellum following X-irradiation at birth. Mol. Chem. Neuropathol. 20, 45–57.

Guelman, L.R., Zieher, L.M., Zorrilla Zubilete, M.A., Dopico, A.M., 1996. Activities of monoamine oxidase-A and -B in adult rat cerebellum following neonatal X-irradiation. Neurosci. Res. 25, 97–100.

Guelman, L.R., Zorrilla Zubilete, M.A., Rios, H., Dopico, A.M., Zieher, L.M., 2000. GM1 ganglioside treatment protects against long-term neurotoxic effects of neonatal X-irradiation on cerebellar cortex cytoarchitecture and motor function. Brain Res. 858 (2), 303–311.

Guelman, L.R., Zorrilla Zubilete, M.A., Ríos, H., Di Toro, C.G., Dopico, A.M., Zieher, L.M., 2001. Motor, cytoarchitectural and biochemical assessment of pharmacological neuroprotection against CNS damage induced by neonatal ionizing radiation exposure. Brain Res. Protoc. 7, 203–210.

Guelman, L., Zieher, L.M., Fiszman, M.L., 1996. The effect of X-radiation on cerebellar granule cells grown in culture. Ganglioside GM1 neuroprotective activity. Neurochem. Int. 29, 521–528.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59, 1609–1623.

Held, K.D., 1985. Interactions of radioprotectors and oxygen in cultured mammalian cells. I. Dithiothreitol effects on radiation-induced cell killing. Radiat. Res. 101, 424–433.

Hochberg, Y., Tamhane, A., 1987. Multiple Comparisons Procedures. Wiley, New York.

Inouye, M., Kameyama, Y., 1983. Cell death in the developing rat cerebellum following X-irradiation of 3–100 rad: a quantitative study. J. Radiat. Res. 24, 259–269.

Kameyama, Y., Inouye, M., 1994. Irradiation injury to the developing nervous system: mechanism of neuronal injury. Neurotoxicology 15 (1), 75–80.

Kermer, P., Klöcker, N., Bähr, M., 1999. Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo. Cell Tissue Res. 298, 383–395.

Khan, M.A., 1993. Histochemical and ultrastructural investigation of heterogeneous Purkinje neurons in mammalian cerebellum. Cell. Mol. Biol. Res. 39, 789–795.

Landauer, M.R., Davis, H.D., Dominitz, J.A., Weiss, J.F., 1988. Long-term effects of radioprotector WR-2721 on locomotor activity and body weight of mice following exposure to ionizing radiation. Toxicology 49 (2/3), 315–323.

Laverty, R., Taylor, K., 1968. The fluorometric assay of catecholamines and related compounds. Anal. Biochem. 22, 269–279.

Manji, H.K., Drevets, W.C., Charney, D.S., 2001. The cellular neurobiology of depression. Nat. Med. 7 (5), 541–547.

Maxwell, S.R.J., 1995. Prospects for the use of antioxidant therapies. Drugs 49 (3), 345–361.

McDonough, J.H., Mele, P.C., Franz, C.G., 1992. Comparison of behavioral and radioprotective effects of WR-2721 and WR-3689. Pharmacol. Biochem. Behav. 42, 233–243.

Michikawa, M., Lim, K.T., McLarnon, J.G., Kim, S.U., 1994. Oxygen radical-induced neurotoxicity in spinal cord neuron cultures. J. Neurosci. Res. 37, 62–70.

Ratan, R.R., Murphy, T.H., Baraban, J.M., 1994. Oxidative stress induces apoptosis in embryonic cortical neurons. J. Neurochem. 62, 376–379.

Richardson, J.S., 1999. Neuroprotective agents. Phys. Med. Rehabil. Clin. N. Am. 10 (2), 447–461.

Santini, V., Giles, F.J., 1999. The potential of amifostine: from cytoprotectant to therapeutic agent. Haematologica 84, 1035–1042.

Schull, W.J., Norton, S., Jensh, R.P., 1990. Ionizing radiation and the developing brain. Neurotoxicol. Teratol. 12, 249–260.

Singh, A., Singh, H., Henderson, J.S., 1990. Radioprotection by ascorbic acid, desferal and mercaptoethylamine. Methods Enzymol. 186, 686–696.

Tabachnik Schor, N.F., 1987. Adjunctive use of ethiofos (WR-2721) qith free-radical-generating chemotherapeutic agents in mice: new caveats for therapy. Cancer Res. 47, 5411–5414.

Tandler, C.J., Ríos, H., Pellegrino de Iraldi, A., 1997. Differential staining of two subpopulations of Purkinje neurons in rat cerebellum with acid dyes. Biotec. Histochem. 72 (5), 231–239.

Weiss, J.F., Landauer, M.R., 2000. Radioprotection by antioxidants. Ann. New York Acad. Sci. 899, 44–60.