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Research Report

Hippocampal-related memory deficits and histological damage induced by neonatal ionizing radiation exposure. Role of oxidative status.

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ABSTRACT

Ionizing radiations induce oxidative stress on target tissues, mainly through the generation of reactive oxygen species (ROS). However, there are few data available on the behavioral effects of moderate doses of ionizing radiation. The aim of the present work was to evaluate the performance of adult rats irradiated at birth in different hippocampal-dependent behavioral tasks and to establish a relationship with the oxidative status and histological changes in rat hippocampus (Hip). Male Wistar rats were irradiated with 5 Gy of X rays between 24 and 48 h after birth. Thirty days later, rats were subjected to open field, object recognition and inhibitory avoidance tasks. In addition, oxidative status markers as well as protein kinase C (PKC) activity and histological changes were assessed in control and irradiated Hip. Results show an impairment in recognition and habituation memories in 30-day-old animals exposed to neonatal ionizing radiation, both at short- (ST) and at long-term (LT), whereas an improvement in associative memory was observed at ST. In addition, histological alterations were observed in irradiated Hip. Although an increase in ROS levels and PKC activity were found in irradiated Hip, no changes in the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were observed. Taken together, our results support the hypothesis that an increased PKC activity, induced by neonatal ionizing radiation on rat Hip, could play a role in the generation of an imbalance between ROS levels and antioxidant systems and might underlie radiation-induced hippocampal histological damage as well as the Hip-dependent behavioral changes found in irradiated rats.

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1. Introduction

Although radiotherapy treatment in human subjects has been widely used as an effective tool to kill tumor cells, it might produce harmful effects to surrounding healthy tissues (Sezen et al., 2008). For this reason, the assessment of ionizing radiation effects on normal brain in experimental models could provide useful data to ensure the delivery of appropriate doses to the brain with a rational criterion.

It is well known that ionizing radiations induce oxidative stress on target tissues, mainly through the generation of reactive oxygen species (ROS) (Halliwell, 1992; Sathyasaikumar et al., 2007). Superoxide radical anion, hydrogen peroxide and hydroxyl radical are the most relevant ROS, which are capable of producing oxidative attack to biological molecules. In normal physiological conditions, ROS are spontaneously generated and efficiently scavenged by the endogenous antioxidant system, which includes both enzymatic (superoxide dismutase (SOD), catalase (CAT)) and non-enzymatic (carotenoids, glutathione (GSH), vitamin E) antioxidant molecules, in order to prevent cellular damage by maintaining the balance between oxidants and antioxidants (Halliwell, 1992). However, certain environmental challenges can increase the production of ROS, which may override the cellular antioxidant defenses, leading to oxidative stress (Sathyasaikumar et al., 2007).

Oxidative stress has been implicated in a number of neurodegenerative diseases (Cassarino and Bennett, 1999) as well as in neurotoxicity models (Goodlett and Horn, 2001). Likewise, we and others have demonstrated that oxidative stress is triggered after ionizing radiation exposure (Riley, 1994; Guelman et al., 2003, 2004, 2005; Limoli et al., 2004; Di Toro et al., 2007).

The abundance of poly-unsaturated fatty acids and the low-level of defensive mechanisms, together with the high oxygen consumption, make the brain more susceptible to oxidative damage than other organs. In particular, previous studies of this laboratory have shown that cerebellum (CE) is a highly radiosensitive structure (Dopico and Zieher, 1993; Guelman et al., 1993, 1996, 2001). It was found that ROS levels have been altered in rat CE of adult rats neonatally exposed to ionizing radiations (Di Toro et al., 2007). Moreover, the endogenous antioxidant GSH was increased (Di Toro et al., 2007) and an exogenous ROS scavenger showed to be effective in the prevention of radiation-induced histological and motor alterations, not only *in vivo* (Guelman et al., 2003), but also *in vitro* (Guelman et al., 2004, 2005). However, neither ROS levels nor antioxidant enzymes activities have been evaluated in other Central Nervous System (CNS) structures, such as the hippocampus (Hip) of rats irradiated at birth.

The doses used in the present work are too high to be used therapeutically in humans at the equivalent developmental stage that the newborn rat (week 24 to 40 of human fetal development, e.g., third trimester of pregnancy or a premature baby); in fact, chemotherapy (not radiotherapy) treatments are preferred in children under 3 years (Baranov et al., 1995). However, it would not be discarded the possibility of an accidental irradiation of a pregnant woman or a premature child which could lead to an impairment of cognitive functions (Kisková and Šmajda, 2008). In consequence, the irradiation of the neonatal rat Hip becomes clinically relevant (Monje and Palmer, 2003; Fukuda et al., 2004; Naylor et al., 2008).

Since Hip is a structure involved in various memory processes that are essential for creating new memories, and lesions to the Hip have shown to impair learning and memory in a variety of behavioral paradigms (Purves, 2004; Jarrard et al., 2004), it would be suggested that ionizing radiation could induce damage to the Hip which might result in hippocampal-related behavioral alterations. Interestingly, since oxidative damage to the brain has been associated with behavioral disorders (Liu et al., 2003; Mecocci et al., 2004; Harman, 1992) and we have reported that neonatal ionizing radiation exposure, through the generation of cerebellar ROS, induces a CE-related behavioral impairment (Caceres et al., 2009), it might be suggested that injury to Hip might underlie radiation-induced disturbances in hippocampal-related behaviors.

Protein kinase C (PKC) represents a family of serine/threonine kinases that plays an important role in signal transduction, as it is involved in the control of numerous cellular processes. Moreover, multiple lines of research implicate PKC in activity-dependent memory formation (Knapp and Klann, 2002; Palumbo et al., 2007). Therefore, the establishment of a correlation between behavior, PKC activity and ROS levels in the irradiated Hip could provide data about ionizing radiation-induced mechanism of damage.

There are few data available on the behavioral effects of moderate doses of ionizing radiations (Raber et al., 2004a; Di Toro et al., 2007; Manda et al., 2007, 2008). In a previous paper, we found that exposure of neonatal rats to 5 Gy of ionizing radiations induced an impairment in spatial memory, together with a decrease in anxiety-like behavior, mainly related with a cerebellar cytoarchitecture damage (Caceres et al., 2009). Therefore, novel data about neonatal rat hippocampal susceptibility to ROS-mediated ionizing radiation damage would be obtained using hippocampal-dependent behavioral tasks, such as open field habituation (Vianna et al., 2000), object recognition (Ennaceur and Delacour, 1988) and inhibitory avoidance (Roozendaal et al., 2002) tests, both at short term (ST, 1 h intertrial interval) or at long term (LT, 24 h intertrial interval).

In consequence, considering a link between ionizing radiation exposure, PKC activity, oxidative stress and behavioral output, the aim of the present work was to evaluate the performance of neonatally irradiated animals in different hippocampal-dependent behavioral tasks and to establish a relationship with the oxidative status and histological changes in rat Hip after neonatal ionizing radiation exposure.

2. Results

2.1. *Behavioral parameters*

2.1.1. *Open field task (OF)*

There were no significant differences in locomotor activities (number of lines crossed) between the irradiated and control groups when the animals were placed for the first time in the

OF (first session, Figs. 1a and b). However, while a significant reduction in the number of lines crossed by control rats in the second session was observed, both at ST (Fig. 1a) and at LT (Fig. 1b), irradiated rats did not reduce the number of lines crossed when re-exposed to the open field (Fig. 1a, ST: $F_{1,\ 30}=22{,}8$, $p<0.001$, Control (C): first session vs. second session, $p<0.001$; Irradiated (Rx): first session vs. second session, NS; Fig. 1b, LT: $F_{1,\ 28}=5{,}46$, $p<0.05$, C: first session vs. second session, $p<0.05$; Rx: first session vs. second session, NS).

2.1.2. *Object recognition task (OR)*

In the OR task, a decrease in total exploration time of the objects was observed in irradiated animals, both at ST (Fig. 2a) and at LT (Fig. 2b), in recognition and testing sessions (ST: $F_{1,28}=21.31$, $p<0.001$, recognition session: C vs. Rx, $p< 0.01$; testing session: C vs. Rx, $p<0.05$; LT: $F_{1,28}=16.79$, $p<0.001$, recognition session: C vs. Rx, $p<0.01$; testing session: C vs. Rx, $p<0.05$). Moreover, latency to objects was significantly increased in irradiated animals in the recognition session, both at ST and at LT (Fig. 3a, ST: $F_{1,30}=6{,}56$, $p<0.05$; recognition session: C vs. Rx, $p<0.05$, testing session: C vs. Rx, NS; Fig. 3b, LT: $F_{1,27}=5{,}4$, $p<0.05$; recognition session: C vs. Rx, $p<0.01$, testing session: C vs. Rx, NS). Finally, while control animals explored more the novel object (N) than the familiar (F) one at LT, exploration time of each object was similar in irradiated animals, both at ST and at LT (Fig. 4a, ST: $F_{1,32}=0.03$, NS; Fig. 4b,

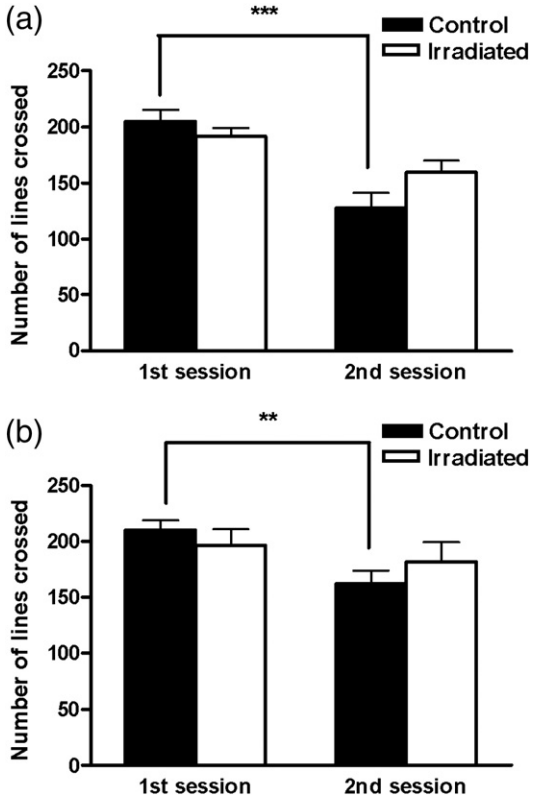

Fig. 1 – Number of lines crossed in the open field task. Filled bars: Control rats; open bars: Irradiated rats. (a) ST; (b) LT. $^{**}p<0.01$ and $^{***}p<0.001$ respect to the first session, respectively. Data are mean of the number of lines crossed ± SEM.

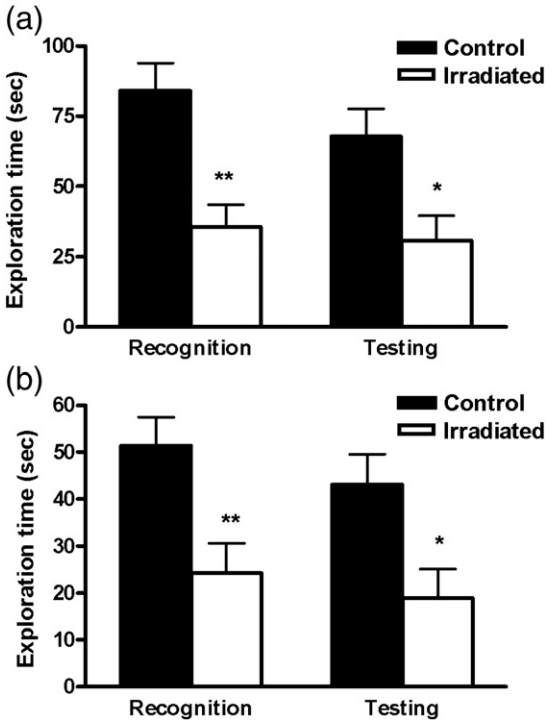

Fig. 2 – Exploration time in the object recognition task. Filled bars: Control rats; open bars: Irradiated rats. (a) ST; (b) LT. $^{*}p<0.05$ and $^{**}p<0.01$ respect to the control, respectively. Data are mean of total exploration time, in seconds, ± SEM.

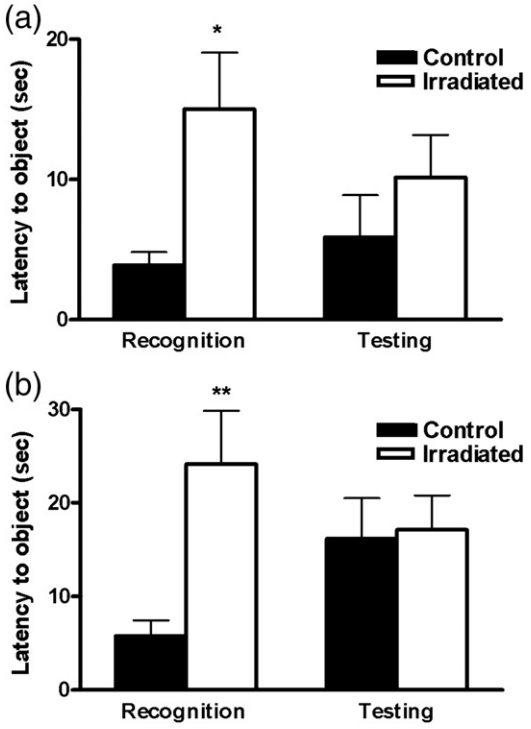

Fig. 3 – Latency to object in the object recognition task. Filled bars: control rats; open bars: Irradiated rats. (a) ST; (b) LT. $^{*}p<0.05$ and $^{**}p<0.01$ respect to the control, respectively. Data are mean of latency to object, in seconds, ± SEM.

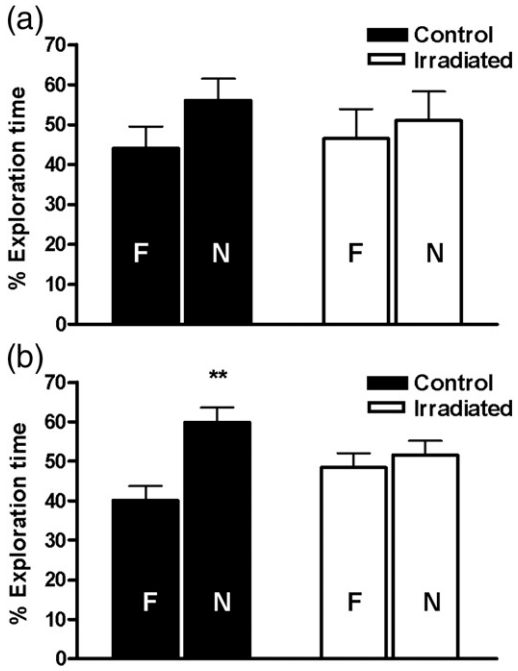

Fig. 4 – Percent of exploration time of novel and familiar object in the object recognition task. Filled bars: Control rats; open bars: Irradiated rats. F: familiar object; N. novel object. (a) ST; (b) LT. **$p<0.01$ respect to the familiar object. Data are mean of the percent of total exploration time ± SEM.

LT: $F_{1,26}=7$, 77, $p<0.01$; Control: N vs. F, $p<0.001$; Irradiated: N vs. F, NS).

2.1.3. *Inhibitory avoidance task (IA)*

Fig. 5 shows results of IA experiments. Irradiated animals significantly increased the latency to enter the dark compartment when compared to control animals at ST (Fig. 5a, C: 24.7 ± 5.7; Rx: 51.6 ± 8.2, $p<0.05$). No differences were observed at LT (Fig. 5b).

2.2. *Biochemical parameters*

Fig. 6a shows a significant increase in ROS levels of irradiated 30-day-old Hip (C: 0.135 ± 0.009; Rx: 0.250 ± 0.005, $p<0.001$). In addition, PKC activity was increased in irradiated Hip (Fig. 6b, C: 44.33 ± 2.33; Rx: 71.37 ± 11.00, $p<0.05$). In contrast, no changes in hippocampal CAT (Fig. 6c) and SOD (Fig. 6d) activities were found in irradiated animals.

2.3. *Histological findings*

Histological sections stained with hematoxylin and eosin showed a mild and focalized decrease in the number of neurons in hippocampal CA1 pyramidal cell layer of irradiated rats when compared to control animals sections, probably induced by neonatal ionizing radiation exposure. Although a decrease in cell number was also observed in dentate gyrus (DG) layer of irradiated rats, the difference was not statistically significative (Figs. 7a and b). When the thickness of the layers was measured, no changes were observed in the Hip of irradiated animals, both in CA1 and DG layers (CA1: C: 73.39 ± 1.06 μm; Rx: 71.34 ± 1.47 μm, NS; DG: C: 74.61 ± 7.92 μm; Rx: 90.26 ± 5.04 μm, NS, data not shown).

Pyknotic and condensed isolated nuclei were observed in irradiated Hip CA1 region, suggesting cellular damage. No signs of pyknosis were found in DG layer (In % of pyknotic cells: CA1: C: 4.5 ± 1; Rx: 8.87 ± 0.97, $p<0.01$, DG: C: 0.0165 ± 0.0052; Rx: 0.029 ± 0.015, NS, data not shown). To define the type of cell with nuclear changes in CA1 region, brain sections were stained with specific neuronal (NeuN, 1:1000) and glial (GFAP, 1:500) antibodies. As shown in Fig. 8, pyknotic and condensed nuclei observed in irradiated animals correspond to strong NeuN+ nuclear labeling, while the GFAP+ corresponds to the few astrocytes localized mainly in *striatum radiatum* area. Astrocyte immunostaining was not observed in CA1 hippocampal layer, demonstrating that pyknotic cells observed in irradiated animals might correspond to neuronal bodies located in the CA1 hippocampal area (Fig. 8).

3. Discussion

Results show that an impairment in recognition and habituation memory was induced in 30-day-old animals exposed to neonatal ionizing radiation, both at ST and at LT, whereas an improvement in associative memory was induced at ST. Although increases in PKC activity and ROS levels were observed in irradiated Hip, no changes in the activities of antioxidant enzymes SOD and CAT were found. Finally, mild and focalized histological changes were observed in irradiated Hip.

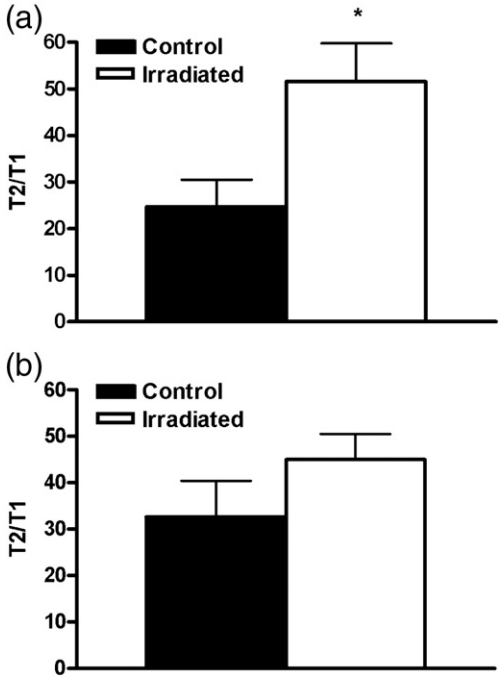

Fig. 5 – Latency to enter the dark compartment in the inhibitory avoidance task. Filled bars: Control rats; open bars: Irradiated rats. (a) ST; (b) LT. *$p<0.05$ respect to the control. Data are mean of the latency to enter the dark compartment (ratio T2/T1) ± SEM.

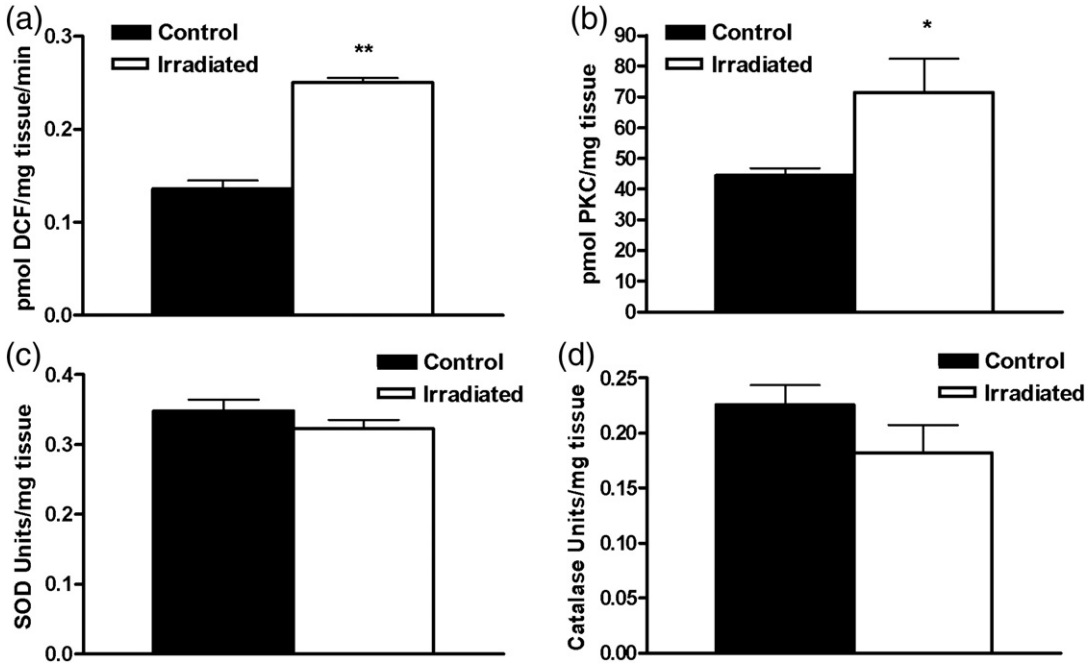

Fig. 6 – Levels of hippocampal oxidative status markers and PKC activity. Filled bars: Control rats; open bars: Irradiated rats. (a) ROS levels; (b) PKC activity; (c) CAT activity; (d) SOD activity. * $p<0.05$ and ** $p<0.01$ respect to the control, respectively. Data are mean of pmol DCF/mg tissue/min (a), pmol PKC/mg tissue (b), U SOD/mg tissue (c) or U CAT/mg tissue (d), ± SEM.

A reduced locomotor activity was observed in control rats during the second exposure to the OF when compared to the first session, in contrast to the lack of change observed in irradiated rats. This difference suggests that, whereas control rats were capable to habituate to the environment, irradiated rats failed to acclimate to it, supporting Park et al. (2001) observations in a model of injury to the Hip. Similarly, in a mutant mice model of Alzheimer's disease characterized by hippocampal dysfunction, a failure to habituate to the environment was found (Deacon et al., 2009). Moreover, prenatally irradiated mice showed impairment in OF performance, as reported by Minamisawa and Hirokaga (1996). It might not be ruled out that the lack of reduction in locomotor activity observed in irradiated rats resulted from an increased activity during the second exposure to the OF, instead of a non-habituation to the device. However, since there were no differences in the number of lines crossed between control and irradiated rats in the first exposure, it would be suggested that it is more likely that habituation memory impairment has been induced to irradiated rats.

It has been suggested that OR depends on the integrity of the Hip (Schröder et al., 2003; de Lima et al., 2006). Present data support this observation, since we found both OR memory impairment and hippocampal damage in rats exposed neonatally to ionizing radiations.

At LT, control animals showed a significant preference for the new object, whereas irradiated rats did not exhibit any object preference, suggesting an impairment in OR memory as reported by Clark et al. (2000) in a model of rats exposed to radio-frequency. Moreover, the decrease in total object exploration time observed in irradiated animals, as well as the increase in the latency to object, both at ST and at LT, would be related to a decreased emotional reactivity, as observed in models of cerebellar dysfunction induced by mechanical (Bobée et al., 2000) or physical agents (Caceres et al., 2009).

Ionizing radiation triggers the release of mediators such as ROS, which are able to function as signaling molecules that might modify memory processes and could be considered either beneficial or neurotoxic. Indeed, Rola et al. (2004) found impairment in IA performance of young mice irradiated with 10 Gy of ionizing radiation, suggesting a neurotoxic role for ROS. On the contrary, the improvement in IA performance observed at ST in the present work in animals irradiated with a moderate dose of ionizing radiations (5 Gy) could be related to the apparent beneficial effects of ROS, supporting Thiels et al.'s (2000) results in a model of transgenic mice that overexpress extracellular SOD.

Interestingly, it has been reported that ROS are capable of inducing PKC activation which, in turn, has been reported to be involved in memory processing (Knapp and Klann, 2002; Jung et al., 2004). Moreover, there seems to be an interactive and feed-forward relationship between PKC and ROS, whereby PKC activation induces ROS production and ROS also can activate PKC pathways (Inoguchi et al., 2000; Dai et al., 2006; Adiga and Nair, 2008). Therefore, the finding of an increase in ROS levels at 30 days post-irradiation – usually observed from hours to several days after acute exposure – would suggest that the radiation-induced increase in PKC activity could be the responsible for inducing the subsequent ROS increase.

The dependence of LT memory on protein kinase A activation and the dependence of ST memory on PKC activity have been postulated by different authors (Izquierdo et al., 2002; Quevedo et al., 2004; Vianna et al., 2000). The improvement in ST memory without changes in LT memory in the IA task, together with the increase in PKC activity found in irradiated animals, reinforce this hypothesis, supporting

Izquierdo et al. (2000) results, who suggest that ST and LT memory may involve separate pathways.

Radiation-induced injury to the Hip has also been observed by Rola et al. (2004) and Czurkó et al. (1997). However, the dose used by those authors was two-fold of that used in the present study (9–10 Gy vs. 5 Gy), and a 50% of lethality was observed in those studies, in contrast to no deaths found in the present work. Therefore, it would be suggested that a sublethal damage might underlie the hippocampal sensitivity to radiation injury (Rodrigues Siqueira et al., 2005).

In adult mice (Raber et al., 2004b) or rats (Erkal et al., 2006; Sezen et al., 2008) exposed to 10 Gy, little damage to hippocampal CA1 layer was induced, suggesting that adult brain is more resistant to radiation injury than developing CNS (UNSCEAR, 1993). In contrast, in the few reports in which immature animals were used, greatest damage was induced to hippocampal CA1 layer in prenatal (Schmitz et al., 2005), neonatal (Czurkó et al., 1997) or 21-day-old mice (Rola et al., 2004) irradiated with 3 to 8 Gy, supporting present data, in which significative changes on cell number were induced to CA1, while cell number in DG layer was unchanged. Moreover, cells with pyknotic nuclei, found only in CA1 layer, have been identified as neurons, suggesting selective neuronal damage.

Conflicting results on the antioxidant enzymatic systems are available in the literature. Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. Abou-Seif et al. (2003) and Sezen et al. (2008) showed decreased brain SOD and CAT levels in irradiated adult rats, while Otsuka et al. (2006) and Zhu et al. (2007) show an increase in antioxidant enzymes activities. Since no significant changes in hippocampal SOD and CAT activities have been observed in neonatally irradiated animals, it would

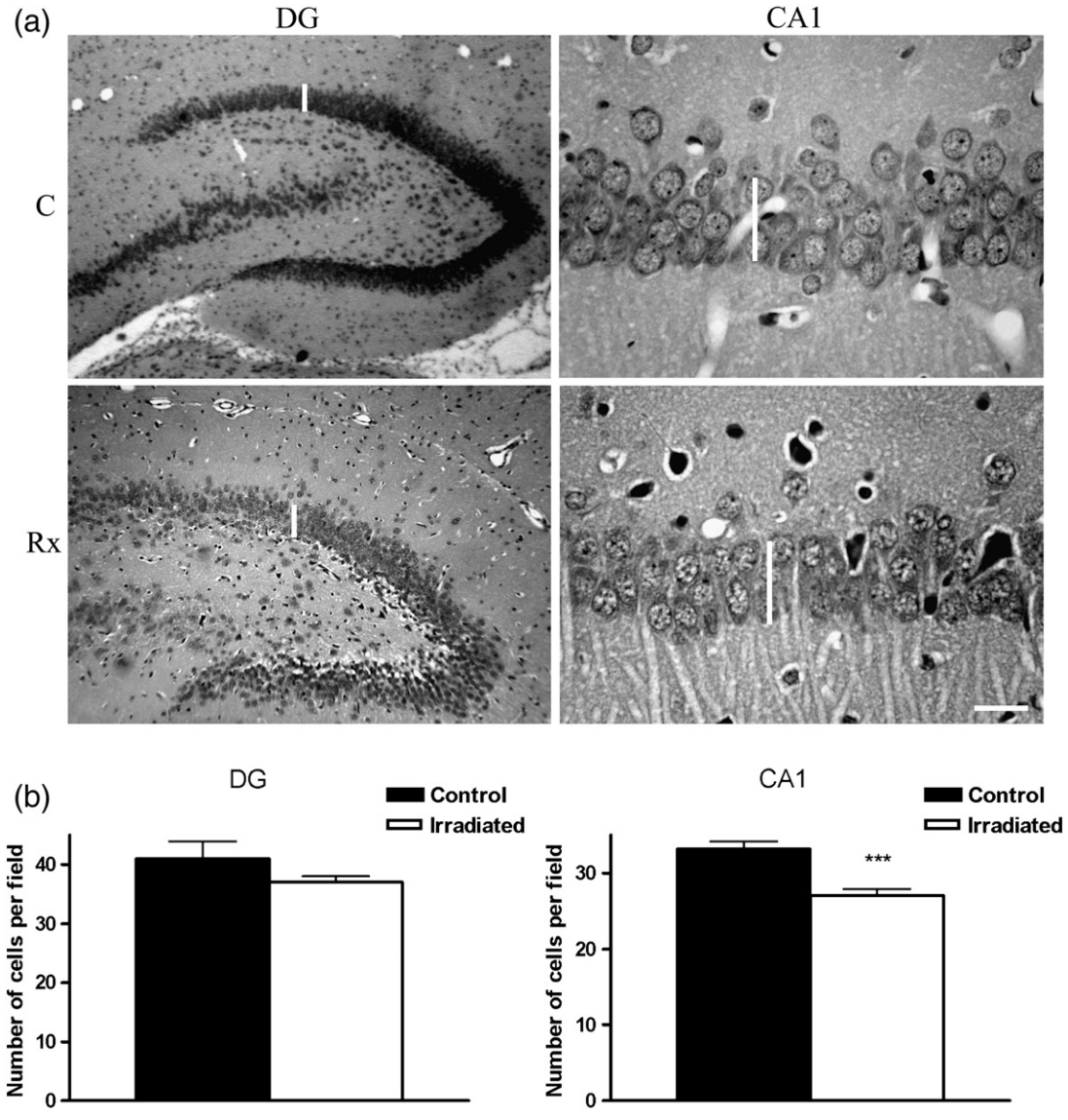

Fig. 7 – Hippocampal coronal hematoxylin–eosin sections at 4.5× and 40× of control and neonatally irradiated 30-day-old rats (a). Ionizing radiation exposure induces a significative change in the number of neuronal cells in CA1 region (b). CA1: Pyramidal CA1; DG: Dentate gyrus. Vertical lines indicate the thickness of the layers. Data are expressed as the number of cells in 60000 μm² for CA1 region and 10000 μm² for DG. Scale bar: 30 μm.

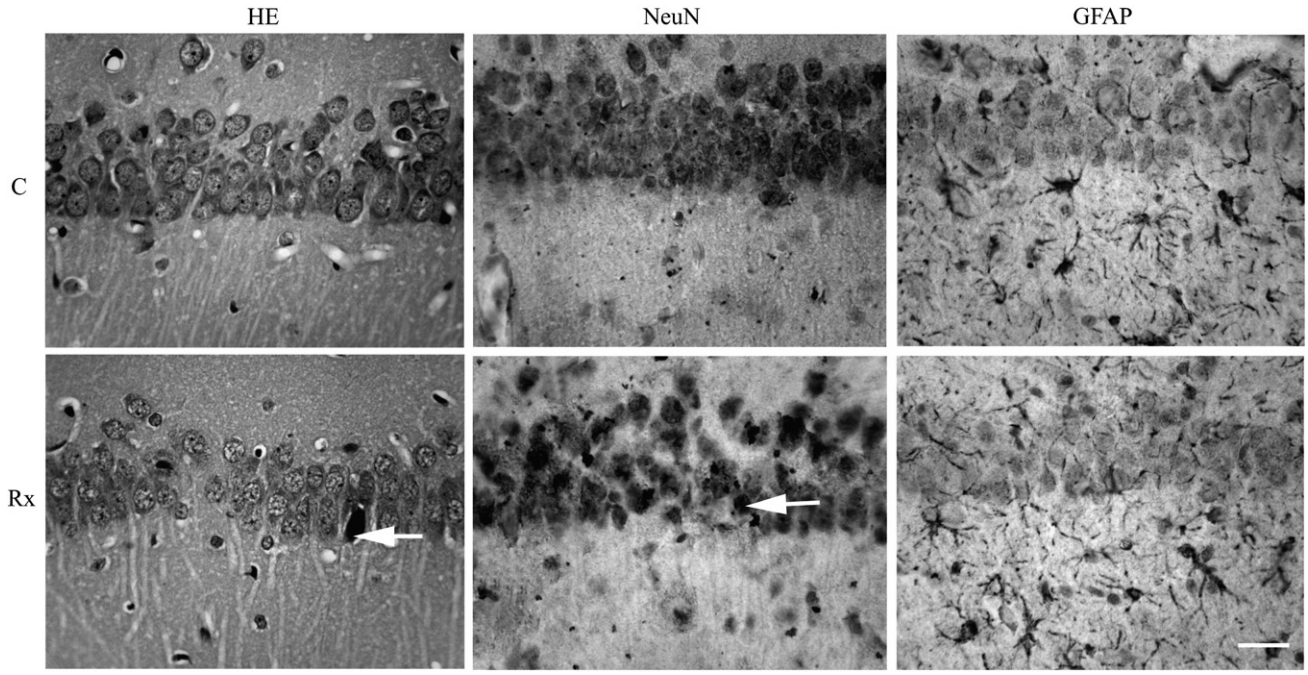

Fig. 8 – Identity of cell type with apparently pyknotic nuclei in CA1 layer, at 40×. HE: hematoxylin–eosin staining; NeuN: neuronal staining; GFAP: glial staining. Arrows point pyknotic cells. Scale bar: 30 μm.

not be discarded that a change in non-enzymatic antioxidants, such as GSH, could have produced. The increase in cerebellar GSH content observed in the irradiation model supports this hypothesis (Di Toro et al., 2007).

Since Erkal et al. (2006) have shown that antioxidant enzymes levels changed immediately after irradiation and at 48 h returned to control values, it cannot be discarded that the unchanged antioxidant enzymes activities observed in the present work could be preceded by an early increase and subsequent return to control values at 30 days. Besides, it would be possible that the unchanged antioxidant enzymes activities observed in the present work were related to the dose of radiation delivered, since a marked decrease in antioxidant enzymes levels was reported by Erkal et al. (2006) and Sezen et al. (2008) using higher doses, while lower doses might enhance the antioxidant defense system (Otsuka et al., 2006). Finally, although the unchanged levels of antioxidant enzymes in irradiated Hip seem to suggest a resistance of antioxidant enzymes to ionizing radiation damage, the lack of ability of these endogenous antioxidant enzymes to compensate the increase in hippocampal ROS levels could be responsible for the histological and behavioral alterations. Therefore, the major finding reported here is that an impaired oxidant balance has been induced in the irradiated Hip, suggesting a high vulnerability of this brain structure to oxidative stress (Castagne et al., 1999; Candelario-Jalil et al., 2001; Rodrigues Siqueira et al., 2005).

It would not be ruled out that an inflammatory response in the neurogenic region of the hippocampus would be induced, as suggested by Monje and Palmer (2003) in Alzheimer's, Lewy body and AIDS dementia. Moreover, the finding of an increase in interleukines (O'Donnell et al., 2000) and eicosanoids levels (Lonergan et al., 2002) after radiation exposure in different models, supports this hypothesis.

Results presented here suggest that a spectrum of functional and morphological deficits could be induced after human *in utero* irradiation, since it is the human period equivalent to the rodent neonatal stage in which animals were exposed to ionizing radiations in this work. Therefore, it is recommended that care should be taken to avoid radiation exposure of the fetus and, in particular, upon the exposure of prematurely born children to different environmental agents (Altman, 1987). Although therapeutic irradiation is rarely used in these patients, it would not be discarded that an accidental irradiation of a pregnant woman or a premature baby could occur. Therefore, the lack of literature reports involving neonatal animals at moderate doses of radiation supports the use of developmental models as the used in the present work to study radiation effects.

Taken together, our results support the hypothesis that an increased PKC activity, induced by neonatal ionizing radiation on rat Hip, could play a role in the generation of an imbalance between ROS levels and enzymatic scavenging systems and might underlie radiation-induced histological damage to the Hip, as well as Hip-dependent behavioral changes found in irradiated rats.

4. Experimental procedures

4.1. *Animals*

Pregnant females were isolated in a cage a few days before delivery. The day of birth (day 0) was known by daily inspection of the cages. Neonatal male albino Wistar rats were randomly separated into two treatment groups, control (C) and irradiated (Rx) rats. Pups were irradiated or sham-irradiated and kept with their dam until 22 days of age. After

weaning, they were separated and maintained four per cage until 30 days, with food and water *ad libitum*, on 12 h light–dark cycles (lights on at 7 am) at 22±2 °C. Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina) and the protocol was approved by this Committee under resolution 2079/07. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) and the institution has an Animal Welfare Assurance approved by the Public Health Service (PHS) with the assurance number A5801-01. Adequate measures were taken to minimize animal pain or discomfort.

For all experimental procedures, rats of 30 days were used. For biochemical studies, rats of both groups were killed by decapitation at 30 days.

For behavioral studies, two intertrial intervals were used, upon which the animal was re-exposed to the device: in the short-term group (ST), the interval between sessions was 1 h, while in the long-term group (LT), the interval was 24 h.

For biochemical studies, eight animals were used, both in control and irradiated groups. For histological studies, three animals were used, both in control and irradiated groups. For behavioral studies, eight animals were used in control and irradiated groups, using different animals for ST and LT experiments. Six additional animals had to be used in inhibitory avoidance test to reach the criterion of having a SEM of 20%, and therefore fourteen animals were used in this test, both at ST and at LT (total animals: ninety eight).

4.2. *Irradiation procedure*

Only the heads of neonatal male Wistar rats (between 24 and 48 h after birth) were exposed to a single 5 Gy dose of X rays, obtained from a high-energy electron linear accelerator (Mevatron Siemens, 6 MV of photon energy). The dose-rate was of approximately 1 Gy/min, being the total time of radiation exposure approximately 5 min. Radiation energy absorbed into the tissue was approximately 5 J/kg, since 1 Gy is a unit of absorbed dose equivalent to 1 J/kg. Pups were immobilized in a plastic holder and the heads fixed in a plastic frame. The beam of X-rays was collimated upon the head at a distance of 50 cm, and homogenized using acrylic plates. The number of subjects remained unchanged throughout the study, since at the dose of radiation used in the present study, no animals deaths were produced.

Radiation protection was always used by the experimenter.

4.3. *Histology*

4.3.1. *Tissue processing*

Animals were anesthetized with 28% chloral hydrate (0.1 ml per 100 mg of body weight) and perfused intracardially with Ringer's media at 35 °C, followed by 4% paraformaldehyde at pH 7.2. Brains were dissected and fixed for 2 additional hours in the same fixative solution. The tissue was dehydrated and included in paraffin. After that, coronal brain sections containing the hippocampal area were obtained with a Micron microtome (4–6 μm of thickness) and recovered for light microscopy analysis. Some slides were stained with hematoxylin and eosin (HE) and others were used to perform immunocytochemistry using specific antibodies.

4.3.2. *Immunocytochemistry*

It was performed on paraffin brain sections of 4 μm. Endogenous peroxidase was quenched (3% H_2O_2 in PBS 0.1 M) and antigenic rescue was performed in citrate buffer pH 6 at 100 °C in a bath water. Non-specific labeling was blocked using 10% normal goat serum. Sections were incubated overnight at 4 °C with a mouse primary antibody NeuN (1:1000, Sigma) or a mouse primary antibody that recognizes GFAP (1:500, Sigma) overnight at 4 °C. After several washes, the sections were incubated for 2 h at room temperature with secondary antibodies (Biotinylated anti mouse IgG, Vector, diluted 1:300), and followed by incubation with a Biotin Streptavidin (HRP Histo Mark, Caramillo, CA USA). After washing in PBS, sections were developed with AEC substrate kit (Invitrogen Gauttersburg, MD, USA) until staining was optimal as examined by light microscopy (5 min) and photographed with an Axiophot Zeiss microscope, with 4.5× and 40× of magnification.

4.3.3. *Histological morphometry*

The degree of severity of the lesion was determined in irradiated animals and compared with the intact control by measurement of the thickness of the layers and cell number using the software NIH ImageJ 1.40 g (Roy et al., 2005). The slides were coded and the examiner was blinded to treatment group. To ensure uniform sampling, we maintained the septotemporal and mediolateral orientations, and used the positions of blood vessels as landmarks. The values obtained for each parameter in a given animal were averaged to produce a single number. We took the middle of the ectal limb of the DG, as well as the pyramidal cell layer of the CA1 region and counted the number of neurons in a fixed field size (60000 μm^2 for CA1 and 10000 μm^2 for DG). The thickness of the pyramidal cell layer of hippocampal CA1 and the granule cell layer of the DG was measured considering the distance between the most extreme nuclei across the layer.

4.4. *Behavioral testing*

4.4.1. *Open field task (OF)*

Repeated sessions in an OF device can be used to analyze habituation memory. Animals were placed on the left rear quadrant of a 50×50×45 cm open field with a floor divided into 25 equal rectangles by black lines. After a 3-min habituation to the behavioral room, the number of lines crossed was measured on the OF over 6 min sessions. This is a classical measure of locomotor activity. In a second session, at ST or at LT, animals were left to explore the apparatus again for another 6 min and the same measure was recorded to evaluate habituation to the task (Barros et al., 2006). Activity was recorder using a camcorder. To minimize the olfactory stimulus, the floor of the box was cleaned with a 10% ethanol solution between sessions.

Comparison between the behaviors of all groups during the first 6-min period on the first session (when the environment would have maximal novelty) with the behaviors during the 6-

min period on second session (when control animals would normally exhibit acclimation to the environment) was performed.

Habituation to a novel environment is believed to be one the most elementary forms of non-associative learning, known to depend on Hip (Vianna et al., 2000; Barros et al., 2006), in which the repeated exposure to the same environment induces a reduction in the exploratory behavior.

4.4.2. *Object recognition task (OR)*

The object recognition task was performed according to the protocol reviewed by Bevins and Besheer (2006). Object recognition task is a test for visual hippocampal functions (Ennaceur and Delacour, 1988; Bevins and Besheer, 2006) used to assess the recognition memory performance of rodents (Heldt et al., 2007; Clark et al., 2000). Briefly, the task was performed in an apparatus that consists in a painted wood box (50×50×45 cm). *Testing* session was performed at ST or at LT after *training* (intertrial intervals). The duration of each session was 5 min. In the *habituation* session, the rat was placed in the box and allowed to freely explore the apparatus for 5 min.

In the *habituation* session, the rat was placed in the box and allowed to freely explore the apparatus for 5 min.

During the *training* session, the apparatus contained two identical objects, while in the *test* session two dissimilar objects were present: a familiar and a novel one. The session started when a rat was placed in the apparatus facing the wall at the middle of the front segment. At the end of session, the rat was immediately put back in its home cage. Activity was recorded using a camcorder. Object recognition was calculated by the percent of time spent exploring the novel object and the time spent exploring the familiar object in the *test* session.

A rat cannot displace the objects and experiments made in a separate cohort of animals demonstrated that rats had no preference for either object or location in the box.

Exploration time and latency to object were also assessed in training and test sessions. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered exploration. To minimize the olfactory stimulus, the box and objects were cleaned with a 10% ethanol solution between sessions. Different groups of rats were used for each intertrial interval.

4.4.3. *Inhibitory avoidance task (IA)*

The inhibitory avoidance test is a task which measures the memory of an aversive experience through the simple avoidance of a location in which the unpleasant experience occurred. This task relies heavily on the dorsal Hip (Ennaceur and Delacour, 1988; Izquierdo and Medina, 1997). We used an inhibitory avoidance apparatus as described by Deacon et al. (2002) and Roozendaal et al. (2002). Briefly, the apparatus consists of a box (60 cm×60 cm×40 cm), divided into two compartments: one is illuminated, while the other is equipped with a removable cover to allow it to be kept dark. A removable partition divided the two compartments. The floor of the dark compartment consisted of a stainless steel grid at the bottom, through which a continuous current could be delivered.

In the *habituation* session to the apparatus, the rat was placed into the lit box and allowed to freely explore the apparatus. Either after passing 3 times to the dark side or after 3 min remaining in the dark side, the rat was removed from the apparatus. After 10 min, the rat was placed again in the lit side and when it entered the dark, the doors were closed and the rat was retained for 10 s in this side.

In the *training* day, each rat was placed in the lit compartment, facing away form the dark compartment and the latency to move into the dark compartment was recorded. When the rat stepped with all four paws into the dark compartment, a foot shock (1.2 mA, 2 sec duration) was delivered. The rat was then removed from the apparatus and returned to its home cage.

Retention was tested at ST or at LT following a similar procedure, except that no shock was delivered. The ratio between the latency of the rat to move into the dark compartment in the *retention* and the *training* sessions (T2/T1) was taken as a measure of associative memory retention (Deacon et al., 2002). To minimize the olfactory stimulus, the box and objects were cleaned with a 10% ethanol solution between sessions.

4.5. *Biochemical procedures*

4.5.1. *ROS determination*

The levels of hippocampal cerebellar ROS were determined by a method described by Driver et al. (2000). Briefly, hippocampi were homogenized in ice Locke's solution (0.5 mg of tissue/ml). Aliquots of the homogenate were taken and left to warm at room temperature during 5 min. 10 μL of dichloro-fluorescein diacetate (0.97 mg/ml in methanol) were added (10 μM final concentration) and the mixture was incubated at room temperature during 15 min. Finally, the fluorescence was measured at 485 nm (excitation) and 530 nm (emission). A standard curve was performed using oxidized dichloro-fluorescein (DCF). Results were calculated as pmol DCF/mg tissue/min and expressed as mean values ± SEM.

4.6. *Antioxidant enzymes assays*

4.6.1. *SOD activity measurement*

The activity of hippocampal SOD was determined according to McCord and Fridovich (1969). Briefly, hippocampi were homogenized at 10% w/v in a 216 nM, pH 7.8 phosphate-buffered solution and were centrifuged at 900*g* for 10 min. A supernatant alicuot was mixed with 216 nM, pH 7.8 phosphate buffer, 10.7 mM EDTA, 1.1 mM C cytochrome and 0.108 mM xanthine at 25 °C. Reaction started with the addition of 0.1 ml of xanthine oxidase (XO) enzyme solution (2 U/ml). The increase in the absorbance at 550 nm ($A_{550\ nm}$) for 5 min was registered. One unit of SOD is defined as the amount that inhibits the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25 °C in a 3.0-ml reaction volume. Results were calculated as U SOD/mg tissue and expressed as mean ± SEM.

4.6.2. *CAT activity measurement*

The activity of CAT was determined according to Beers and Sizer (1952). Briefly, a 10% w/v hippocampal homogenates were made in 50 mM phosphate buffer and therefore were

centrifuged at 42,000*g* for 15 min. A supernatant alicuot was incubated with 0.036% (w/w) hydrogen peroxide solution (H_2O_2). The time required to decrease the absorbance at 240 nm ($A_{240\ nm}$) from 0.45 to 0.40 absorbance units was registered.

One unit will decompose 1.0 μmole of H_2O_2 per minute at pH 7.0 at 25 °C. Results were calculated as U CAT/mg tissue and expressed as mean ± SEM (Goss et al., 1997).

4.7. *PKC activity*

Hippocampi were quickly dissected in ice and homogenized in 10 mM HEPES (pH 7.5) containing 2 mM EGTA, 0.3 mg/ml DTT, 0.16 mg/ml PMSF and 0.020 mg/ml EDTA. Homogenates were centrifuged at 100,000*g* for 30 min at 4 °C, being the supernatant the soluble fraction of the enzyme. The pellet was resuspended in HEPES containing Nonidet P-40 and maintained in ice for 30 min. After centrifugation at 100,000*g* for 30 min at 4 °C, the obtained supernatant contains the PKC particulate fraction (Genaro and Bosca, 1993). PKC was purified by filtration through a DE 52 column (3.5×0.5 cm). Both fractions were eluted in a buffer containing 120 mM NaCl, 10 mM ß-mercaptoethanol, 0.5 mM EGTA, 10 mM HEPES (pH 7.4) and its activity was assayed by measuring the incorporation of ^{32}P from [^{32}P-γATP] into histone1 (H1). Incubations were performed for 30 min at 37 °C. The reaction was stopped by the addition of 2 mL of ice-cold 5% trichloroacetic acid. The radioactivity retained on GF/C glass-fiber filters after filtration was determined by counting the filters in 2 mL of scintillation fluid (Zorrilla Zubilete et al., 2005). PKC activity was determined after subtracting the incorporation of ^{32}P into H1 in the absence of calcium and phospholipids. Results were calculated as pmol PKC/mg tissue.

4.8. *Statistical analysis*

Significant differences between two groups were determined by the Student test. When more than 2 groups were compared, ANOVA statistical was used and Tukey test was applied for *post-hoc* comparisons. Results are expressed as mean values ± SEM of 8–14 animals. A probability < 0.05 was accepted as significant.

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