RESEARCH COMMUNICATION

Comparison of Protective Effects of L-Carnitine and Amifostine on Radiation-induced Toxicity to Growing Bone: Histopathology and Scintigraphy Findings

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Abstract

Purpose: The aim of the present study was to evaluate the radioprotective efficacy of L-carnitine (LC) in growing bones in comparison to amifostine. Materials and Methods: Sixty two-week-old Wistar albino rats were randomly assigned to six equal groups: Group 1, control (CONT); Group 2, irradiation alone (RT); Group 3, amifostine plus irradiation (AMI+RT); Group 4, L-carnitine plus irradiation (LC+RT); Group 5, amifostine alone (AMI); Group 6, L-carnitine alone (LC). The rats in the AMI+RT, LC+RT and RT groups were irradiated individually with a single dose of 20 Gy to the left femur. LC (300mg/kg) and amifostine (200mg/kg) were applied 30 min before irradiation. The animals were scanned for bone area, mineral content and bone mineral density (BMD) by DEXA and the 99mTc methylene diphosphonate uptake ratio (MUR) was calculated by bone scintigraphy. Histopathological analysis of bone and cartilage was also carried out after euthanasia. Results: Pretreatment with LC or amifostine reduced the radiation-induced damage in growing bone (p=0.007 and p=0.04 respectively) and in the epiphysial cartilage (p=0.002 and p=0.015 respectively). The protective effect of LC was similar to that of amifostine on both growing bone and on the epiphysial cartilage. The mean left-femur BMD values were significantly higher in the LC+RT (p=0.02) and AMI+RT (p=0.01) groups than in the RT group. but did not differ with the two protective agents. Pretreatment with AMI (p=0.002) and LC (p=0.01) improved the MUR. Conclusions: L-carnitine is equally as effective as amifostine at protecting growing bone against single dose irradiation damage.

Keywords: Amifostine - growing bone - irradiation - L-carnitine - radioprotection

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Introduction

Radiation therapy is an important treatment modality in pediatric oncology. One of the most important dose-limiting factors of irradiation is the permanent shortening and deformity of bones that can occur in the irradiation field in growing children (Paulino, 2004). This significant growth arrest may be caused by fractionated doses of 15 Gy and above in young children and can occur with doses as low as 10 Gy in children under 1 year of age (Goldwein, 1991; Robertson et al., 1991). The reduction of radiation-induced bone damage has been considered in a number of different dose-fractionation schemes. However, growth arrest is not completely eliminated even with dose-fractionation approaches, and the necessity of multiple sessions of anesthetic sedation further limits its clinical usage (Eifel et al., 1990; Alheit et al., 1998; Damron et al., 2000). Prophylactic use of radioprotectants prior to fractionated irradiation is an alternative strategy for further reduction of bone damage in these patients.

The effect of ionizing radiation is primarily mediated through the action of free radicals, which can cause damage to DNA, proteins, and lipids (Riley, 1994). Therefore, antioxidative defense mechanisms are responsible for much of the radiation damage (Weiss and Landauer, 2000). Amifostine (S-2{3-aminopropylamino-ethylphosphorothioic acid; Ethyol; WR-2721) is a prodrug that is converted in vivo by alkaline phosphatase to an active sulfhydryl compound (WR-1065). This substance selectively protects normal cells from antineoplastic drug toxicity by scavenging free radicals, by donating hydrogen ions to free radicals, by depleting oxygen, and by binding to active derivatives of antineoplastic agents (Williams et al., 1983; Kouloulias et al., 2004). Our previous studies showed substantial radioprotective effects of amifostine on lung and kidney (Uzal et al., 2004; Kaldir et al., 2008; Caloglu et al., 2009). The effectiveness of amifostine as a radioprotective agent in irradiated bone has also been shown in earlier studies (Spadaro et al., 2003; Damron et al., 2004). However, use of amifostine has been reported to be accompanied by undesirable side-effects including nausea, vomiting, sneezing, hot flashes, mild somnolence,

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hypocalcaemia and hypotension (Kligerman et al., 1984; Andreassen et al., 2003).

L -Carnitine (3-hydroxy-4-trimethylammoniumbutyric acid) (LC) is a small water-soluble molecule that facilitates the transfer of long-chain fatty acids into the mitochondria of skeletal muscle and cardiomyocytes, where they undergo beta-oxidation (Vanella et al., 2000). LC prevents the formation of ROS produced by the xanthine/xanthine oxidase system and thus decreases damage to the cell membrane. LC is obtained mostly from the diet or can be given exogenously. It can also be synthesized endogenously by skeletal muscle, heart, liver, kidney, and brain and is also a relatively well-tolerated and safe (Fritz and Arrigoni-Martelli, 1993; Bertelli et al 1994). The radioprotective effect of LC has been shown in earlier studies (Mansour, 2006; Altas et al., 2006; Ucuncu et al., 2006; Kocer et al., 2007; Caloglu et al., 2009).

To the best of our knowledge, no study has yet investigated the efficacy of LC in prevention of radiation-induced growing bone damage. Our aim was therefore to evaluate radioprotective effects in irradiated growing bones in comparison with those afforded by amifostine.

Materials and Methods

Animals and experimental design

All animal experiments adhered to the guidelines of the Institutional Animal Ethics Committee. Infant rats were housed with their mothers until four weeks-old, and then were housed in rat cages with ad libitum access to a standard rodent diet and tap water, with a 12:12-hr artificial light cycle, mean temperature 21± 2°C, and mean humidity 55±2%. When they had reached two weeks of age, all animals were randomly assigned into six groups of ten rats each, for the following treatments:

Group 1: Control (CONT), normal saline alone, injected with normal saline (200mg/kg) by intraperitoneal injection (i.p.) 30 minutes before a sham irradiation;

Group 2: Irradiation alone (RT), injected with normal saline (200mg/kg) by i.p. 30 minutes before irradiation;

Group 3: Amifostine before irradiation (AMI+RT), injected with amifostine (200mg/kg) by i.p. 30 minutes before irradiation 4;

Group 4: LC before irradiation (LC+RT), injected with LC (300mg/kg) by i.p. 30 minutes before irradiation. The selection of the 30-min interval between LC administration and exposure to radiation was based on our previous study on animals 13;

Group 5: Amifostine alone (AMI), injected with amifostine (200mg/kg) by i.p. 30 minutes before a sham irradiation;

Group 6: LC alone (LC), injected with LC (300mg/kg) by i.p. 30 minutes before a sham irradiation.

All experimental procedures were performed on anesthetized rats. Anesthesia was maintained with ketamine and xylazine (35mg/kg BW and 3mg/kg BW, i.m. for infant rats and 50mg/kg BW and 5mg/kg BW, i.m. for adults) during irradiation and scintigraphic examination. The follow-up period was 6 months. During follow-up, all rats were monitored by the veterinary care staff.

Irradiation

The rats in AMI+RT, LC+RT and RT groups were irradiated individually with a single dose of 20 Gy. Doses of irradiation were given with 6 MV photon at a depth of 1 cm through an anterior 2.5×2cm single portal (with a 1cm bolus) covering the left-femur, using a Linear Accelerator treatment unit (Varian 2100 CD, Varian Inc., Palo Alto, CA,USA) at a source skin distance of 100 cm. The rats were anesthetized and then fixed onto a 20×30cm blue Styrofoam treatment couch (Med-Tec, Orange City, IA) in a prone position. Correct positioning of the fields was controlled for each individual rat using a therapy simulator (Mecaserto-Simics, Paris, France). Special dosimetry was done for the irregular fields. The dose homogeneity across the field was ±5%. After irradiation, the animals were closely observed until recovery from anesthesia. The CONT, AMI, and LC groups received an equal field sham irradiation.

Bone mineral analysis

The animals were scanned for bone area, mineral content, and bone density by DEXA, (Hologic QDR 4500, Hologic, Waltham, MA, USA), equipped with rat whole body scan software. The scan field size was 12×8cm, resolution was 0.025×0.012cm and scan speed was 7mm/sec. Data output for bone mineral content (BMC) (g), two-dimensional projected area (cm^2), and bone mineral density (BMD, g/cm^2) were recorded.

Bone scintigraphy

Bone scintigraphy was obtained 3 hours after intravenous administration of 3mCi of 99m Tc-labeled methylene diphosphonate (99mTc-MDP). A gamma camera (Orbiter, Siemens Corp, Iselin, NJ, USA) equipped with high resolution collimator was used. A total of 250K or more counts were accumulated over a period of 10 min per view. Regions of interest on each limb and background were selected in order to quantify the bone scan. Counts of radionuclide uptake were obtained from the midshaft of the femur and from the soft tissue area. The average pixel counts of regions were obtained; and MDP uptake ratios (MUR) were calculated.

Euthanasia

The rats were euthanized 6 months after the radiation therapy by decapitation, under anesthesia using ketamine and xylazine in combination.

Histopathological analysis

The left legs of the rats were fixed in 10% buffered formaldehyde for 24 hours. The proximal femurs with surrounding soft tissues were then dissected and decalcified in 10% formic acid for 24 hours. After processing the tissues in alcohol, all tissues were embedded into paraffin and five micrometer thick sections were cut and stained with hematoxylin-eosin. A pathologist examined each slide under a light microscope (Nikon E400, Japan) three times in a blinded manner. Since, to our knowledge, there has been no similar study in the literature, we formed our own grading method based on examination of the pathologies in the bone and cartilage tissues.

The proximal femur specimens included the proximal diaphysis, epiphysis, and metaphysis. The articular cartilage was not taken into consideration. The pathologies observed in the epiphysial cartilage were noted and graded from 0 to 3, where 0 meant no damage, 1 meant normal thickness of the epiphysial plate with only slight disarrangement in the chondrocyte rows, 2 meant increased thickness in the epiphysial plate with moderate disarrangement in the chondrocytes and mild cellular atypia in chondrocytes, and 3 meant moderate to severe atypia of chondrocytes, showing irregular clumping with thickening of the cartilaginous tissue of the epiphysis and loss of cartilaginous tissue in some parts of the epiphysial plate, indicating early irregular bone formation. Bone pathologies were also graded from 0 to 3, where; 0 meant no pathology, 1 meant slight thickening of the bony trabeculae, 2 meant thickening of trabeculae with or without medullary fibrosis, and slight atypia in the osteoblasts, and 3 meant prominent thickening of the trabeculae and occasional degenerative areas, with or without medullary fibrosis, and moderate to severe osteoblastic atypia.

Statistical analysis

The data, expressed as mean ± S.D, were analyzed using standard statistical methods (Statistica version 7 program). One-way analysis of variance (ANOVA) was used for statistical comparisons between the groups. Spearman's rank correlation test was used to measure the degree of association between the histopathological and DEXA analysis. The differences were considered significant when probability was less than 0.05.

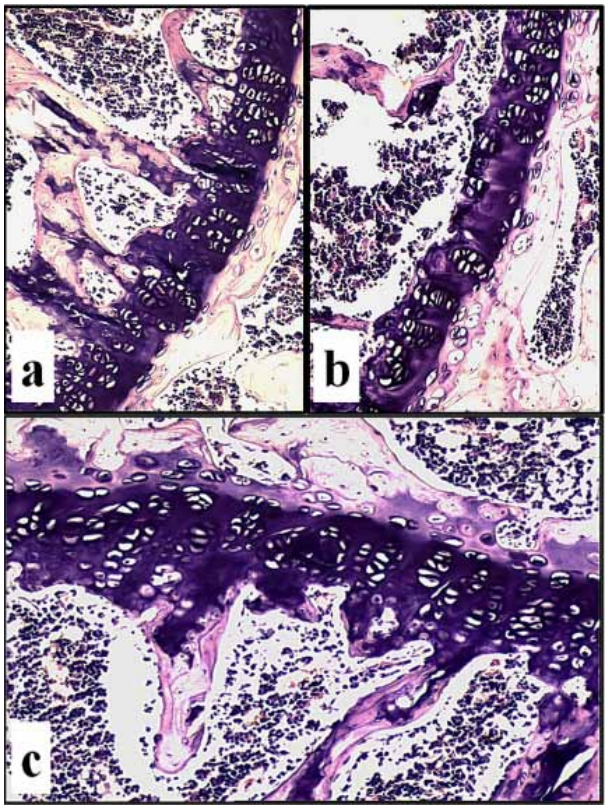

Figure 1. Histology of Epiphysial Cartilage. Rats after treatment with amifostine (a) and L-carnitine (b) and the control (c) (H&E x 100)

Results

Histopathologic and radionuclide imaging analyses were made on 50 rats. Ten rats died during the follow-up period. The distributions of deaths in groups were as follows: 1 death in CONT group, 4 in RT, 1 in AMI+RT, 1 in LC+RT, and 3 in AMI . There were no deaths in the LC group. These rats were excluded from the analysis and histopathologic evaluation was not conducted.

The epiphysial cartilage and bone damage are summarized for each group in Table 1. Radiation-induced growing bone damage was significantly higher in the RT group than in the CONT group ($p<0.0001$). Pretreatment with LC or amifostine reduced the radiation-induced damage in growing bone ($p=0.007$ and $p=0.04$ respectively) compared to the RT group. However, there was significant difference between CONT group and both the LC+RT ($p=0.01$) and the AMI+RT ($p=0.001$) groups. The protective effect of LC was similar to amifostine in the growing bone.

The epiphysial cartilage was histopathologically normal in CONT, AMI and LC groups (Figure 1). The epiphysial cartilage damage significantly increased in the RT ($p<0.0001$) (Figure 2a), LC+RT ($p<0.0001$) (Figure 2b) and AMI+RT ($p<0.0001$) (Figure 2c) groups compared with the CONT group. Epiphysial cartilage damage was significantly reduced in the LC+RT ($p=0.002$) and the AMI+RT ($p=0.01$) groups compared to the RT group. The degree of epiphysial cartilage damage was similar in LC+RT and AMI+RT groups.

The mean BMD, bone size, and MUR are shown

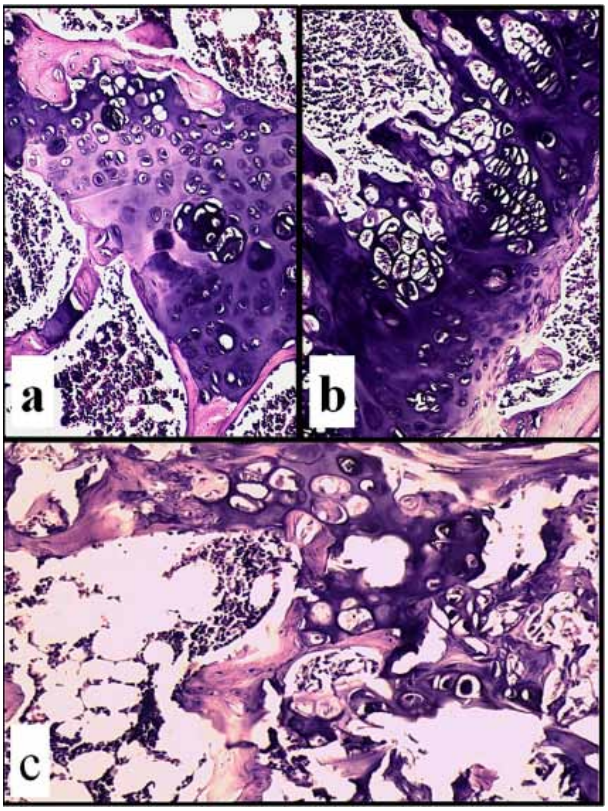

Figure 2. Damage and Thickening in the Epiphysial Cartilage. a) Mild-moderate damage in a LC+RT and b) an AMI +RT rat and c) severe damage after irradiation alone, (H&E x 100)

Table 1. Frequency of Pathological Damage in Each Group According to the Grade of Damage

	CONT (n=9)	RT (n=6)	AMI+ RT (n=9)	LC+RT (n=9)	AMI (n=7)	LC (n=10)	p value*
Epiphysial cartilage damage							
Grade 0	9	-	-	1	7	10	
Grade 1	-	1	3	4	-	-	<0.0001
Grade 2	-	2	6	3	-	-	
Grade 3	-	3	-	1	-	-	
Bone damage							
Grade 0	9	-	4	4	7	10	
Grade 1	-	3	3	4	-	-	<0.0001
Grade 2	-	3	1	1	-	-	
Grade 3	-	-	1	-	-	-	

Data show the number of rats in each group, with percentages given in parentheses. *p value generated from ANOVA; AMI + RT, 200mg/kg, i.p., amifostine 30min prior to irradiation; LC + RT, 300mg/kg, i.p., L-carnitine 30min prior to irradiation; LC, 300mg/kg, i.p., L-carnitine 30min prior to sham irradiation; AMI, 200 mg/kg, i.p., amifostine 30min prior to sham irradiation; RT, normal saline 30min prior to irradiation; CONT, normal saline 30min prior to sham irradiation

Table 2. The Results of Radionuclide Imaging and DEXA Analysis

	CONT (n=9)	RT (n=6)	AMI+ RT (n=9)	LC+RT (n=9)	AMI (n=7)	LC (n=10)	p value*
BMD (g/cm^2)	0.20±0.01	0.16±0.04	0.17±0.02	0.17±0.02	0.18±0.01	0.19±0.01	<0.0001
Bone size (cm^2)	1.51±0.27	0.72±0.22	0.82±0.16	0.84±0.22	1.14±0.16	1.34±0.13	<0.0001
MDP uptake rate	5.19±3.97	28.85±40.34	4.23±3.82	10.14±9.04	3.48±1.53	6.49±2.05	0.02

Data show the number of rats in each group, with percentages given in parentheses. *p value generated from ANOVA; AMI + RT, 200mg/kg, i.p., amifostine 30min prior to irradiation; LC + RT, 300mg/kg, i.p., L-carnitine 30min prior to irradiation; LC, 300mg/kg, i.p., L-carnitine 30 min prior to sham irradiation; AMI, 200mg/kg, i.p., amifostine 30min prior to sham irradiation; RT, normal saline 30min prior to irradiation; CONT, normal saline 30min prior to sham irradiation; BMD, Bone mineral density (g/cm^2); Bone size, two-dimensional projected bone area (cm^2); MUR, 99mTc MDP uptake ratio

Table 3. The Correlations between of Histopathological and DEXA Analysis

L-Femur	BMD (g/cm2)	Bone size (cm2)
Epiphysial cartilage damage	r= -0.39 p= 0.005	r= -0.77 p= <0.0001
Bone damage	r= -0.3 p=0.039	r= -0.56 p=<0.0001

BMD, Bone mineral density (g/cm^2); Bone size, two-dimensional projected bone area (cm^2); r= Spearman's rank correlation coefficient

in Table 2. Left-femur BMD values were significantly decreased in the RT (p<0.0001), LC+RT (p<0.0001) and AMI+RT groups (p<0.0001) compared with the CONT group. The mean left-femur BMD values were significantly higher in the LC+RT (p=0.02) and AMI+RT (p=0.01) groups compared to the RT group. The mean level of BMD was similar in the LC+RT and the AMI+RT groups. Left-femur size decreased after irradiation (CONT: 1.51±0.27 vs RT: 0.72±0.22; p<0.0001). Bone size in LC+RT (0.84±0.22) and AMI+RT (0.82±0.16) groups was higher than in the RT group, but the difference was not significant. As shown in Table 3, epiphysial cartilage and bone tissue damage were correlated with BMD and area values.

An increased L-femur MUR was observed after irradiation (CONT: 5.19±3.97 vs RT: 28.9±40.4; p=0.002). Pretreatment with LC (p=0.01) and AMI (p=0.002) ameliorated this increase in MUR. The mean MUR values were similar in the LC+RT (10.2±9.04), the AMI+RT (4.23±3.82) and the CONT (5.19±3.97) groups.

Discussion

Pretreatment with LC reduced radiation-induced bone and epiphysial cartilage damage to an equal extent as did amifostine, as determined by histopathological findings. Furthermore, both BMD and bone metabolism were ameliorated to the same extent by pretreatment with either LC or amifostine. However, this amelioration of BMD did not restore bone growth to the CONT level. LC and amifostine had moderate effects on bone size; as animals in the LC and AMI groups had significantly decreased bone size compared with animals in the CONT group.

Effects of irradiation, such as immediate or delayed cell death, cellular injury, arrest of cellular division, and abnormal repair, are to be expected in tissues. In developing bone, irradiation is known to affect the immature skeleton by interfering with chondrogenesis and reabsorption of calcified cartilage and bone at the growth plate, but the underlying mechanism of irradiation damage in growing bones is not completely understood (Rubin et al., 1959; Probert and Parker, 1975). This study showed that irradiation clearly caused damage in epiphysial cartilage and bone tissue, and reduced bone mineral density, metabolism, and size. Previous studies have shown that irradiation may create a destructive process, such as an unbalanced situation between osteoclastic and osteoblastic activity. After exposure to radiation, the number of osteoblast and osteocyte cells is reduced, and this also causes a decline in collagen synthesis and alkaline phosphatase activity. Thus, bone matrix formation is impaired and the mineralization process is disturbed

(Williams and Davies, 2006).

LC is a substance that can act as an antioxidant and free radical scavenger (Hagen et al., 2002). In addition, LC has the capacity to control carbohydrate metabolism, to maintain cell membrane structure and cell viability, and it is an essential cofactor in the oxidation of long-chain fatty acids (Athanassakis et al., 2001). We used LC as a possible modulator of radiation-induced toxicity, based on the previous reports. Caloglu et al., (2009) showed that LC ameliorated radiation-induced renal damage in rats. Furthermore, LC increased endogenous antioxidant defense mechanisms, which might have protected the animals from radiation-induced organ toxicity (Mansour, 2006). Altas et al. (2006) showed that LC could improve radiation-induced cochlear damage in guinea pigs. LC also was shown to serve as a protective agent against irradiation-induced lens damage in a rat study by Kocer et al. (2007). The radioprotective properties of LC in delaying the onset and reducing the severity of radiation-induced oral mucositis have also been reported in another animal study (Ucuncu et al., 2006).

To the best of our knowledge, there has not yet been any study on the effects of LC on radiation-induced growing bone and cartilage damage. However, a limited number of studies have suggested that that LC has positive effects on osteoporosis and BMD (Patano et al., 2008). Hooshmand et al. (2008) stated that LC application in rats following ovariectomy significantly increased BMD in tibia, and concluded that LC may also similarly increase BMD by lowering the rate of bone turnover. Benvenga et al. (2001) reported that oral intake of LC increased BMD, and that LC had a positive effect on bone mineralization in hyperthyroid patients. LC has also shown anabolic effects in a few studies using either osteoblasts or bone marrow cells (Benvenga et al., 2004; Colucci et al., 2005).

Several studies have assessed amifostine effects on bone cells (Weiss et al., 1986; Wong et al., 2009). Margulies et al. (1986) reported that chondrocyte and osteoblast cell counts were reduced 47.3 and 31.9%, respectively, after exposure to 20-Gy irradiation, relative to controls ($p<0.004$). Pretreatment with amifostine showed a 24.0 and 30.2% sparing in osteoblast and chondrocyte cell numbers, but this difference was not statistically significant. Damron et al. (2003) noted that although amifostine had little effect on osteoclast numbers, the number of chondroclast profiles was higher in the region of the chondro-osseous junction in limbs that were pretreated with amifostine than in the ones treated with radiation alone. Gevorgyan et al. (2008) observed that radiation significantly impaired clonogenic survival, osteoblast function, and osteoblast-like phenotype. Notably, WR-1065, the active metabolite of amifostine, protected cultured normal osteoblast-like cells from the effects of irradiation. The present study now shows that epiphysial cartilage as well as bone damage can be significantly decreased by pretreatment with amifostine. There was no grade 3 epiphysial cartilage damage in AMI+RT group. Moreover, 44% of animals in AMI+RT group had no histopathologically observable radiation-induced bone tissue damage.

Several studies have focused on growth effects such as loss of limb length, regarding the effects of amifostine on radiation-induced growing bone damage. Damron et al. (2001) reported that a single radiation dose of 25 Gy reduced growth in overall limb length by a mean of 58.8% (range 54.2-66.4%, SD 4.2%) in the treated leg. This difference in limb length was statistically significant between irradiated and non-irradiated 4-week-old rats ($p< 0.0001$). Amifostine administration at a dose of 200 mg/kg prior to irradiation resulted in an insignificant reduction in limb length loss ($p< 0.05$). Moreover, Tamurian et al. (1999) showed that pretreatment with 100 mg/kg of amifostine caused a statistically significant reduction in growth loss due to single dose 12.5-Gy and 17.5-Gy irradiation in weanling rats. In the present study, left-femur size significantly decreased after irradiation. Bone size in the AMI+RT (0.82±0.16) and LC+RT (0.84±0.22) groups was greater than in the RT (0.72±0.22), but the difference was not statistically significant ($p>0.05$).

The pretreatment with LC in the current study significantly reduced the radiation-induced damage in BMD, to an equivalent extent as that seen with amifostine. BMD was significantly decreased after irradiation, similar to results from previous reports. Margulies et al. (2008) showed an irradiation-induced diminution of BMD. Furthermore, Forrest et al. (2002) noted that 20 minutes before 35-Gy radiation, pretreatment with amifostine showed significant ($p<0.05$) preservation of BMD in the rabbit orbital-zygomatic complex, compared with controls. In the present study, BMD was significantly increased in LC-treated as well as in amifostine-treated rats.

Extremely elevated MUR ratios (28.9±40.3) were observed in the RT group rats. Pretreatment with LC or amifostine ameliorated this MUR increase to CONT levels. This result could indicate that pretreatment with amifostine or LC might increase the number of live cells remaining after irradiation, which allows recovery to continue.

However, our study has some limitations. Firstly, the radiation-induced growing bone model was set on a single-dose irradiation, which is different from routine clinical application. In routine practice, irradiation is applied in fractions with the rationale of preventing damage to normal tissues. Because rats in the control group were 2-week-old pups, the procedure was planned in a single dose and radiation-induced growing bone damage was targeted. The impact of fractionated irradiation and LC implementation should also be taken into account in future studies. Secondly, we did not evaluate antioxidant properties of LC at the tissue level. Even though this point may seem to be an important issue, different authors in the literature have previously reported on the antioxidant properties of LC. On the other hand, this initial study aimed to compare the effectiveness of LC and amifostine on radiotherapy-induced growing bone damage using scintigraphic and histopathologic methods. Further studies are necessary to determine the mechanisms of the protection afforded by these compounds, by evaluating markers of oxidative stress in bone.

In conclusion, LC protected the single fraction irradiation induced growing bone damage to an apparently

equal extent as did amifostine. Radiation-induced retardation of bone growth remains a significant clinical side effect for pediatric cancer survivors. It would also be worthwhile to study the effects of LC supplements and amifostine in radiation-treated cancer patients, with the hope of reducing radiation-induced growing bone damage.

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