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Original Article

Effects of ionizing radiation on proteins in lyophilized or frozen demineralized human bone

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ABSTRACT

Objective: The aim was to study the effects of application of ionizing radiation (gamma and electrons) as sterilizing agents at doses of 15 kGy, 25 kGy and 50 kGy, on lyophilized or frozen demineralized bone tissue for use in transplants.
Methods: Five human femoral diaphyses from different donors of musculoskeletal tissue were demineralized and preserved as lyophilized or frozen at −80 °C. The samples were divided into two groups: non-irradiated (control) and irradiated by means of gamma rays or an electron beam. The bone proteins were extracted and used to determine the concentrations of total protein and BMP 2 and 7.
Results: Decreases in total protein and BMP 2 and 7 concentrations were observed. The decreases in total protein concentrations, in comparison with the respective control groups, were significant in the lyophilized and frozen samples that were irradiated at a dose of 50 kGy of gamma radiation and electron beam, with reductions of more than 30%. Significant decreases in the levels of BMP 2 and 7 were also observed at higher doses and especially through use of the electron beam.
Conclusion: The reductions in the concentrations of total proteins and osteoinductive proteins (BMP 2 and 7) were related to the radiation dose, i.e. they increased with higher doses of ionizing radiation type and the type of bone preservation. The largest reductions in concentrations were observed in the bones irradiated by means of an electron beam and at a dose of 50 kGy. However, this type of radiation and this high dose are not usual practices for sterilization of bone tissue.

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Efeitos da radiação ionizante nas proteínas presentes em ossos humanos desmineralizados, liofilizados ou congelados

Palavras-chave:
Osso e ossos
Radiação ionizante
Banco de tecidos
Proteína morfogenética óssea 2
Proteína morfogenética óssea 7

RESUMO

Objetivo: Estudar os efeitos da aplicação das radiações ionizantes (gama e elétrons) como agentes esterilizantes, nas doses de 15 kGy, 25 kGy e 50 kGy, nos tecidos ósseos desmineralizados congelados e liofilizados para uso em transplantes.

Métodos: Cinco diáfises femorais humanas de doadores distintos de tecidos musculoesqueléticos foram desmineralizadas e preservadas como liofilizadas ou congeladas a −80 °C. As amostras foram divididas em grupos não irradiados (controle) e irradiados por raios gama ou feixe de elétrons. As proteínas ósseas foram extraídas e dosadas as concentrações de proteínas totais, BMP 2 e 7.

Resultados: Foi observada diminuição das concentrações de proteínas totais e BMP 2 e 7. A diminuição das concentrações de proteínas totais, quando comparada com o respectivo controle, foi significativa nos grupos de amostras liofilizadas e congeladas e irradiadas na dose de 50 kGy por radiação gama e feixe de elétrons com redução superiores a 30%. A diminuição significativa nas concentrações das BMP 2 e 7 também foi observada nas maiores doses e principalmente por feixe de elétrons.

Conclusão: As reduções nas concentrações das proteínas totais e em proteínas osteoindutoras (BMP 2 e 7) foram relacionadas à dose de radiação, ou seja, aumentam com maiores doses, tipo de radiação ionizante e ao tipo de preservação dos ossos. As maiores reduções das concentrações foram observadas nos ossos irradiados por feixe de elétrons e na dose de 50 kGy. Porém esse tipo de radiação e essa alta dose não são práticas usuais para a esterilização dos tecidos ósseos.

Introduction

There is a growing demand for allogeneic musculoskeletal grafts for orthopedic and dental reconstruction in Brazil. Bone tissue can be highlighted within this, with 21,681 distributions in 2014.

Musculoskeletal tissue banks are organizations that take on the responsibility for selecting donors and obtaining, processing, storing, distributing and performing quality control on tissues.

Bone tissues can be obtained from both living and deceased donors. These musculoskeletal tissues are mainly used in revision arthroplasty procedures on hips or knees, in treating patients with bone tumors, in joint reconstruction for treating ligament injuries, in raising the maxillary sinus in cases of atrophy and in mandibular grafts for placement of dental implants.[1]

The ideal allogeneic material for use as a grafting option should have the following properties: biocompatibility, not causing infection, low immunogenicity, osteoconduction and osteoinduction.

Osteoinduction is an osteogenesis process that promotes recruitment of mesenchymal pluripotent undifferentiated cells and stimulation toward differentiation into bone-forming cells. Bone morphogenic proteins (BMPs) are an important group of glycoproteins extracted from demineralized bone matrix that are responsible for bone induction.[2]

BMPs are classified as a subfamily within the superfamily of transforming growth factor-β (TGF-β). According to the literature, BMPs 2, 4 and 7 have the greatest potential for inducing osteoblastic differentiation from progenitor mesenchymal cells.

Performing a demineralization procedure on cortical bone matrix increases the biological availability of BMPs and makes these grafts osteoconductive.[3] There is a positive correlation between the BMP content that is present in grafts and the degree of osteoconductivity.[4]

There is great concern about ensuring tissue quality and promoting safety among patients who receive homogenous tissues, with regard to transmission of infectious and contagious diseases.

With the aim of eliminating possible contamination, bone tissue donors undergo serological screening and assessment of their histories and social behavior, along with molecular biological tests to detect viral RNA from HIV and HCV, clinical examinations and microbiological control tests. Aseptic techniques are applied during the procedures.[5] However, there is the possibility of contamination with microorganisms during tissue acquisition, processing, preservation and storage.[6]

Many professionals responsible for tissue banks consider that it is important to sterilize biological tissues using an effective method such as ionizing radiation.

Sterilization through ionizing radiation is a method that presents advantages over other methods. It produces only a minimal increase in temperature and does not leave toxic residues, which makes it usable and also a form of final sterilization.[7] However, some authors have concluded that sterilization through ionizing radiation may cause dose-related structural and biological alterations to bone tissues.[5,8]

Different types of bone preservation may affect both protein maintenance and the interaction of ionizing radiation with the tissue. Different doses of ionizing radiation may also influence bone integrity with regard to maintaining the osteoconductive potential of these grafts.

Better knowledge of these parameters may help people in charge of tissue banks in choosing the tissue irradiation and preservation conditions and ensuring the quality of grafts used in transplantations.

The objective of this study was to assess the effects of applying gamma radiation and electron beams produced by ^{60}Co sources and electron accelerators, respectively, at doses of 15 kGy, 25 kGy and 50 kGy to bone tissue that had been demineralized, preserved, frozen and lyophilized. An additional aim was to evaluate the possible alterations to total protein and BMP-2 and BMP-7 concentrations, in order to determine the best dose for ensuring safety with regard to sterility and preservation of the osteoinductive proteins of bone grafts.

Materials and methods

After the project had been approved by the institution's research ethics committee, under no. 238/12, it was started through selection of samples from five femoral diaphyses that had been acquired as bone tissue donations from five different deceased individuals: two males and three females aged between 39 and 65 years (mean of 52). The material had been obtained in accordance with the musculoskeletal bone tissue protocol of our department.

The cortical bone tissue of the femoral diaphyses was ground up into particles and those that were smaller than 1 mm were selected. From each diaphysis, 48 g of the bone tissue particles thus selected was divided into 16 groups (3 g per sample), thus producing a total of 80 samples coming from the five donors.

Subsequently, part of the sample (42 g) was demineralized in 0.5 N HCl solution in a glass beaker, in the proportions of 50 ml of solution to one gram of tissue, for 90 min at a temperature of 18 °C ± 2 °C. An orbital mechanical stirrer was used at low rotation to keep the solution constantly mixed during the entire demineralization process.[3] Some of the sample was then lyophilized or frozen.

The demineralized bone tissue was divided into two groups: lyophilized or frozen.

The samples in these two groups were irradiated at doses of 15, 25 and 50 kGy using two radiation sources: electron accelerator (electron beam) or cobalt-60 (gamma ray). The samples were classified as: LIO 15; 25; 50 AE, LIO 15; 25; 50 γ, CG 15; 25; 50 AE; CG 15; 25; 50 γ. Two control samples of non-irradiated demineralized tissue were also created.

The samples were irradiated at the Institute of Energy and Nuclear Research (IPEN). The samples of the groups were irradiated with gamma rays from ^{60}Co sources from a multipurpose irradiator at a dose rate of 0.00138 kGy/s; or with an electron beam from an industrial electron accelerator at a dose rate of 3.92 kGy/s. To avoid temperature variations, the lyophilized and frozen samples were irradiated at 4 °C and −70 °C, respectively.

To extract BMPs and total proteins from the bone matrix, a buffer containing guanidine–HCl was used as described in the following: 0.5 g of tissue from each sample was added to 5 ml of fresh solution of 4 M guanidine–HCL and 0.05 M Tris–HCL at pH 6.0, with a mixture of protease inhibitors: 5 mM benzamidine–HCL; 0.1 M 6-aminohexanoic acid; 0.5 mM phenylmethylsulfonyl fluoride; 5 mM N-ethylmaleimide. This was kept under agitation at a temperature of 4 °C for 24 h. The tubes were then centrifuged at 697 × *g* for 5 min. The supernatant was removed and placed in a second tube.

Another 5 ml of fresh solution of 4 M guanidine–HCL, 0.05 M Tris–HCL and the mixture of protease inhibitors was added in order to resuspend the sediment. The samples were kept under agitation at a temperature of 4 °C for 5 h.[3] After this, the samples were again centrifuged and the supernatant was added to the second tube described above. The supernatant solutions were then divided into aliquots and frozen at −80 °C.

The total proteins and BMPs 2 and 7 were quantified using the Bradford and ELISA methods, respectively. The assays were performed in triplicate and the result was taken to be the arithmetic mean from the assays.

The results from each group were compared with their respective controls, and the controls were compared with each other.

The statistical analysis on the data consisted of comparison of the results by means of the one-way ANOVA method followed by the Tukey test for statistical differences of $p < 0.05$.

Results

Table 1 and Fig. 1 show the quantification of total proteins in mg/g of demineralized bone tissue and the graded reductions in mean concentrations in the irradiated groups in relation to their respective non-irradiated controls. Statistically significant alterations were observed in the following groups: LIO 50 γ (31.9%); LIO 50 γ AE (35.6%); CG 25 γ (18.3%); CG 50 γ (39.8%); CG 15 AE (13.6%); CG 25 AE (18.3%); and CG 50 AE (36.1%).

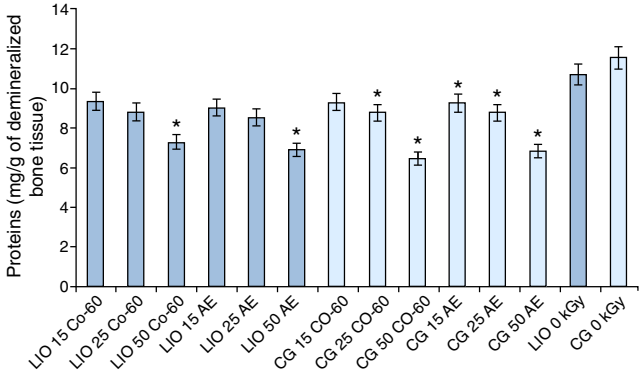

Fig. 1 – Effects of ionizing radiation on the proteins of demineralized bone tissue. Quantification of the total proteins extracted from organic bone matrix in relation to the irradiated and non-irradiated (control) groups. Representation of the results from assaying total proteins per group. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

Table 1 – Decrease in percentage mean concentration of total protein in the different groups studied in relation to the control.

Lyophilized groups	mg/g of demineralized bone tissue	Reduction (%)	Frozen groups	mg/g of demineralized bone tissue	Reduction (%)
LIO control	10.7	–	CG control	11.54	–
LIO 15 γ	9.35	12.5	CG 15 γ	9.29	13.1
LIO 25 γ	8.79	17.8	CG 25 γ	8.74	18.3[a]
LIO 50 γ	7.28	31.9[a]	CG 50 γ	6.44	39.8[a]
LIO 15 AE	9.02	15.7	CG 15 AE	9.24	13.6[a]
LIO 25 AE	8.52	20.3	CG 25 AE	8.74	18.3[a]
LIO 50 AE	6.89	35.6[a]	CG 50 AE	6.83	36.1[a]

[a] Indicates a statistically significant difference in relation to the control ($p < 0.05$).

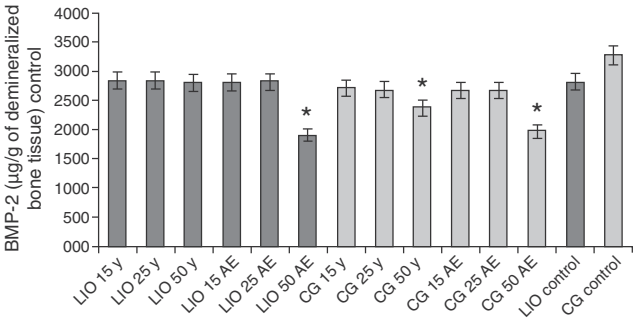

Fig. 2 – Effects of gamma and electron-beam radiation on the concentrations of concentrations of BMP-2 per gram of demineralized bone tissue in the groups studied. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

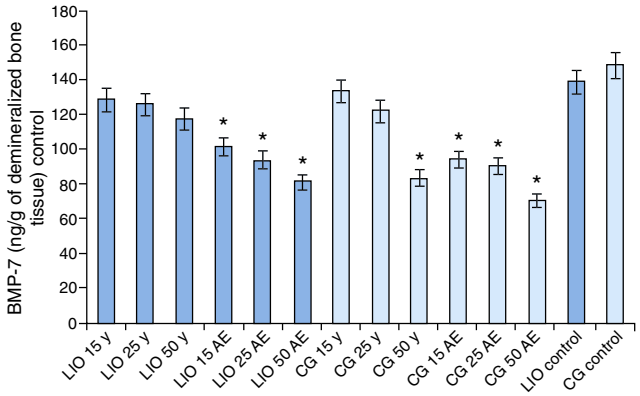

Fig. 3 – Effects of gamma and electron-beam radiation on the concentrations of concentrations of BMP-7 per gram of demineralized bone tissue in the groups studied. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

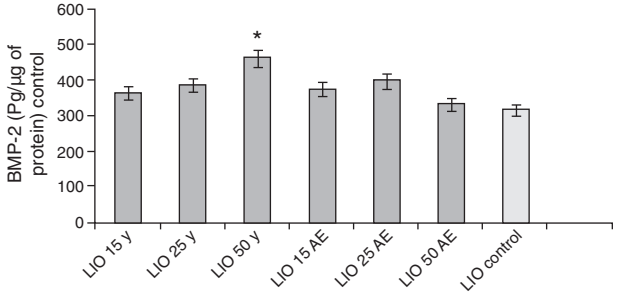

Fig. 4 – Relationship between the concentrations of BMP-2 and specific total proteins in each lyophilized tissue group: non-irradiated and irradiated with gamma and electron-beam radiation. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

BMPs 2 and 7 were quantified with the aim of analyzing the effects of the ionizing radiation and the possible alterations to the concentrations of these proteins. The results obtained regarding concentrations were correlated in terms of grams of demineralized bone tissue and grams of total protein.

The results obtained from the groups analyzed are presented in Tables 2 and 3. These show the results regarding mean concentrations of BMP-2 and BMP-7, in µg/g and ng/g, respectively. In the lyophilized and frozen groups, we noted percentage reductions in the mean concentrations of BMP-2 and BMP-7 in the different irradiated groups in relation to the non-irradiated controls.

Table 2 and Fig. 2 show the effects of the ionizing radiation on the concentrations of BMP-2 in the groups studied. Statistically significant changes, with reductions in mean concentrations in relation to the respective control, were observed in the following groups: LIO 50 AE (32.6%); CG 50 γ (27.2%); and CG 50 AE (39.4%).

Table 3 and Fig. 3 show the effects of ionizing radiation on the concentrations of BMP-7, in the different groups studied. Statistically significant changes, with reductions in the mean concentrations of BMP-7 in relation to the respective control, were observed in the following groups: LIO 15 AE (26.9%); LIO 25 AE (32.4%); LIO 50 AE (41.7%); CG 50 γ (44.1%); CG 15 AE (36.4%); CG 25 AE (39.3%); and CG 50 AE (52.6%).

Figs. 4 and 5 show the mean concentrations of BMP-2 in relation to the total proteins assayed in the irradiated and non-irradiated groups. There were statistically significant changes in the groups LIO 50 γ and CG 50 γ in relation to the control.

Figs. 6 and 7 show the mean concentrations of BMP-7 in relation to the total proteins assayed in the irradiated and non-irradiated groups. There were statistically significant changes

Table 2 – Record of the BMP-2 assays in the groups studied. Reduction in concentration of BMP-2 in the irradiated groups as a percentage in relation to the non-irradiated control.

Lyophilized groups	BMP-2 (μg/g of demineralized bone tissue)	Reduction (%)	Frozen groups	BMP-2 (μg/g of demineralized bone tissue)	Reduction (%)
LIO control	2.82	–	CG control	3.27	–
LIO 15 γ	2.84	0	CG 15 γ	2.72	16.8
LIO 25 γ	2.83	0	CG 25 γ	2.7	17.4
LIO 50 γ	2.81	0.3	CG 50 γ	2.38	27.2[a]
LIO 15 AE	2.82	0	CG 15 AE	2.67	18.3
LIO 25 AE	2.82	0	CG 25 AE	2.66	18.6
LIO 50 AE	1.9	32.6[a]	CG 50 AE	1.98	39.4[a]

[a] Indicates a statistically significant difference in relation to the control ($p < 0.05$).

Table 3 – Reduction in concentration of BMP-7 in the irradiated groups as a percentage in relation to the non-irradiated control.

Lyophilized groups	BMP-7 (ng/g of demineralized bone tissue)	Reduction (%)	Frozen groups	BMP-7 (ng/g of demineralized bone tissue)	Reduction (%)
LIO control	138.66	–	CG control	148.2	–
LIO 15 γ	128.42	7.4	CG 15 γ	133.29	10.1
LIO 25 γ	125.46	9.5	CG 25 γ	122.03	17.6
LIO 50 γ	117.33	15.4	CG 50 γ	82.85	44.1[a]
LIO 15 AE	101.25	26.9[a]	CG 15 AE	94.2	36.4[a]
LIO 25 AE	93.78	32.4[a]	CG 25 AE	89.97	39.3[a]
LIO 50 AE	80.77	41.7[a]	CG 50 AE	70.18	52.6[a]

[a] Indicates a statistically significant difference in relation to the control ($p < 0.05$).

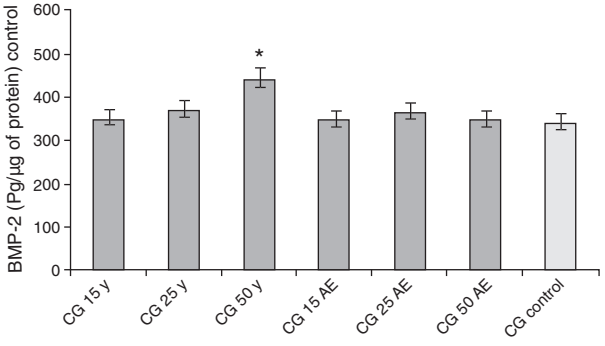

Fig. 5 – Relationship between the concentrations of BMP-2 and specific total proteins in each frozen tissue group: non-irradiated and irradiated with gamma and electron-beam radiation. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

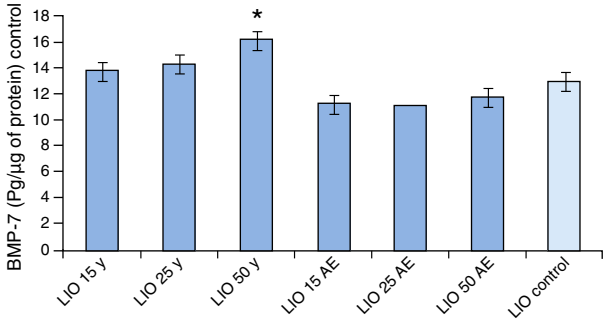

Fig. 6 – Relationship between the concentrations of BMP-7 and specific total proteins in each lyophilized tissue group: non-irradiated and irradiated with gamma and electron-beam radiation. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

in the groups LIO 50 γ, CG 15 AE, CG 25 AE and CG 50 AE in relation to the respective controls.

Discussion

Increased use of musculoskeletal tissue grafts in reconstructive surgery, along with the aim of promoting greater safety in transplantations and in tissue quality, has led tissue banks to seek better techniques for processing, preservation and sterilization.

Gamma radiation is the type most used in tissue banks and has been shown to be an effective method for ensuring final sterilization of biological tissues. However, there have been reports of deleterious effects on the mechanical and biological properties of tissues, depending on the radiation dose applied.[5]

Radiation through an electron beam is a promising option for the tissue sterilization process and it has some advantages in comparison with gamma radiation, such as its capacity for use through a rapid radiation transfer system. Its main disadvantage is the low power of penetration into materials that electron beams present, which may make it difficult or

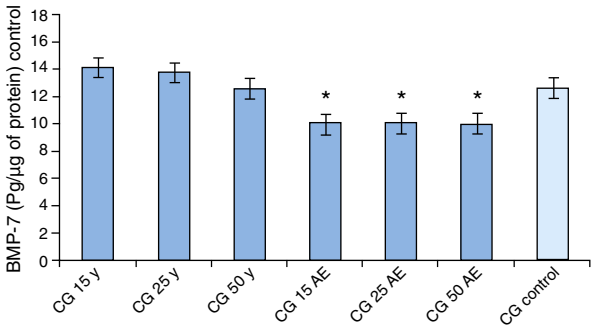

Fig. 7 – Relationship between the concentrations of BMP-7 and specific total proteins in each frozen tissue group: non-irradiated and irradiated with gamma and electron-beam radiation. (*) indicates a statistically significant difference in relation to the control ($p < 0.05$).

impossible to perform irradiation, depending on the structure and density of the tissues.

The activity and concentration of BMPs and growth factors that form part of TGF-β are essential for effective osteoinduction of bone grafts. However, these may become degraded during the process of sterilization through ionizing radiation and may therefore compromise the bone transplantation.[9]

The protein quantification technique is an important parameter, because osteoinduction and osteogenesis are functional processes of bone grafts and depend quantitatively on the existence of bone proteins and growth factors. There is a positive correlation between the BMP content present in grafts and osteoinductivity.[4,10]

There are some studies with divergent results regarding the effects of gamma radiation on the osteoinductive potential of demineralized bone grafts. In 1988, Munting et al.[11] concluded that osteoinduction was totally inhibited through gamma radiation doses greater than 20 kGy. This study was conducted through implantation of demineralized bone tissue into the muscles of rats and rabbits. However, all the grafts were irradiated at room temperature, which might explain the compromised quality of the tissue.

In contrast, other authors[12,13] irradiated demineralized bone tissue using gamma radiation under temperature conditions that were controlled through using dry ice and obtained promising results. This reinforces the hypothesis that bone tissue that is irradiated at low temperatures is less susceptible to radiation. Wientroub and Reddi[13] concluded that a standard dose (25 kGy) of gamma radiation did not alter the osteoinductive potential of demineralized bone tissue that was implanted in muscle tissue in rats, and that higher doses (30–50 kGy) even increased this property.

Likewise, Glowacki[14] reported in 2005 that demineralized bone tissue irradiated with doses between 20 kGy and 40 kGy maintained 80% of their osteoinductive activity. In 2002, Dziedzic-Goclawska reported results found using frozen bone tissue at a temperature of −72 °C that was irradiated with doses of 35 kGy to 50 kGy and concluded that there were no differences in osteoinductive potential, in comparison with non-irradiated bone tissue. However, lyophilized preserved tissue that was irradiated at room temperature at the same doses was completely reabsorbed and lost the capacity to produce osteoinduction.

Through the results obtained from the effects of the ionizing radiation on the concentrations of osteoinductive proteins in the present study, as shown in Table 2 and Fig. 2, we observed that there were significant reductions in BMP-2 in relation to the non-irradiated control, only in the groups irradiated at doses of 50 kGy (LIO 50 AE; CG 50 γ; and CG 50 AE). The largest reductions (greater than 32%) were found in the groups irradiated using an electron accelerator. At the doses of 15 and 25 kGy, under any of the conditions, we did not observe any dose-dependent variation.

The results from assaying BMP-7, as shown in Table 3 and Fig. 3, show that there were significant reduction in relation to the respective controls in all the groups irradiated using an electron accelerator (LIO 15 AE; LIO 25 AE; LIO 50 AE; CG 15 AE; CG 25 AE; and CG 50 AE) and in the frozen group with gamma ray irradiation at a dose of 50 kGy (CG 50 γ). In the groups in which the reduction was not significant, we were able to see tendency toward a dose-dependent decrease (from 7 to 18%).

In comparing the effects from the radiation in the lyophilized and frozen groups studied, although the results from the frozen groups were not statistically significant, they showed a larger reduction in the concentrations of total proteins (13.1–36.1%), BMP-2 (16.8–39.4%) and BMP-7 (10.1–52.6%), in relation to the lyophilized groups (12.5–35.6%; 0.0–32.6%; and 7.4–41.7, respectively). This evidence can be explained by the greater indirect biological effects of ionizing radiation on tissues that present greater presence of water, through radiolysis on the water, which generates larger quantities of free radicals and consequently produces possible damage to the tissues.[15]

In Figs. 4 and 5, a significant increase in the mean concentration BMP-2 per gram of protein can be seen at the gamma radiation dose of 50 kGy in both the frozen and the lyophilized groups. In Fig. 6, it can be seen that there was a significant increase in the mean concentration of BMP-7 per gram of protein in the lyophilized and irradiated groups at the dose of 50 kGy. Fig. 7 shows that there were significant reductions in the mean concentrations of BMP-7 per gram of protein in the samples from the frozen and irradiated groups that received electron beams at the doses of 15, 25 and 50 kGy. This was due to the different effects of ionizing radiation on the concentrations of total proteins and specific proteins (BMP 2 and 7).

Thus, the effects of ionizing radiation with regard to causing damage to bone grafts depend mainly on two factors: (1) the irradiation conditions (radiation dose, dose rate, type of ionizing radiation and radiation temperature); and (2) the physical state of the samples, especially the quantity of water present in the bone tissues.[15]

Conclusion

Ionizing radiation at high doses (50 kGy) caused significant reductions (between 35% and 52%), in the concentrations of total protein and osteoinductive proteins (BMP 2 and 7). It was also observed that radiation consisting of an electron beam caused effects that were more deleterious than those

of gamma radiation, with regard to the quantities of bone proteins studies. However, these conditions are not usually applied to sterilization of bone tissues.

Under the sterilization conditions usually applied to bone tissues (15–25 kGy, using gamma rays), the reductions in the concentrations of the osteoinductive proteins (BMP-2 and BMP-7) were less than 20%.

Conflicts of interest

The authors declare no conflicts of interest.

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