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EFFECTS OF IONIZING RADIATION ON PROTEINS IN DEMINERALIZED, LYOPHILIZED OR FROZEN HUMAN BONE

Uri Antebi[1], Rodrigo P. Guimarães[2] and Monica B. Mathor[3]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
uri@usp.br

[2] Faculdade de Ciências Médicas da Santa Casa de São Paulo (FCMSCSP)
Rua Dr Cesário Motta Júnior, 61
01221-020 São Paulo, SP
clinicaguimaraes@gmail.com

[3] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
mathor@ipen.br

ABSTRACT

The aim is the study of the application of ionizing radiation (gamma and electron) as sterilizing agents at doses of 15 kGy, 25 kGy and 50 kGy, the demineralized bone tissue frozen and freeze-dried for use in transplants. Five human femoral diaphysis of different donors demineralized bone tissues were preserved as lyophilized or frozen at – 80ºC. The samples were divided into non-irradiated groups (control) and irradiated by gamma rays or electron beam. The bone proteins were extracted and used to determine the concentrations of total protein, BMP 2 and 7. It was observed a decrease in total protein concentrations, and BMP 2 and 7. The decrease in total protein concentrations, as compared to respective control groups was significant in the lyophilized and frozen samples irradiated at a dose of 50 kGy gamma radiation and beam electrons with greater than 30% reduction. The significant decrease in the levels of BMP 2 and 7 were also observed in higher doses and especially by electron beam. The reductions in the concentrations of total protein and osteoinductive proteins (BMP 2 and 7), were related to the radiation dose, i.e., increase with higher doses of ionizing radiation type and the type of preservation of the bones. The largest reductions in concentrations were observed in bone irradiated by electron beam and at a dose of 50 kGy. But this type of radiation and this high dose are not usual practice for the sterilization of bone tissue. Keywords: demineralized bone tissue, ionizing radiation, Tissue Bank, BMP 2, BMP 7, bone proteins.

INTRODUCTION

There is a growing demand for musculoskeletal allografts requests for orthopedic and dental reconstructions in Brazil, highlighting the bone tissue distributions with 21,681 in 2014 [1]. Banks of musculoskeletal tissues are organizations that are responsible for the selection of donors, harvesting, processing, storage, distribution and quality control of the tissues. The bone tissues are obtained from deceased or living donors. Musculoskeletal tissues are mainly used in hip arthroplasty and knee revisions in the treatment of patients with bone tumors patients, the joint reconstruction for treatment of ligament injuries in the maxillary sinus lifting with atrophy and grafting in the mandible for dental implant placement [2].

The ideal material for allogenic grafting is used as an alternative should have the following properties: biocompatibility, do not cause infection, low immunogenicity, is osteoconductive

and osteoinductive. Osteoinduction is a process that promotes the recruitment of pluripotent stem cells (mesenchymal cells) and stimulation for differentiation into bone forming cells. An important class of glycoproteins, extracted from demineralized bone matrix consists of bone morphogenetic proteins (BMPs), responsible for the bone induction [3].

BMPs are classified as a subfamily within the superfamily of growth factors and transformation-β (TGF-β), and according to the literature, BMPs 2, 4 and 7 has a higher potential in inducing osteoblast differentiation from mesenchymal progenitor cells. The demineralization procedure in cortical bone matrix increases the bioavailability of BMPs making these grafts osteoindutores [4]. There is a positive correlation between the contents of BMPs present in the grafts and osteoinductivity [5].

There is intense concern to ensure the quality of tissues and promote the safety of patients receiving allogenic tissue in relation to the transmission of infectious diseases. In order to eliminate possible contamination, it is performed in the donor bone tissue, serological screening, assessment of the historical and social behavior, as well as molecular biology tests for viral RNA detection of HIV and HCV, clinical and microbiological controls, besides being applied aseptic techniques during the procedures [6]. However, there is a possibility of contamination with micro-organisms during harvesting, processing, preservation and storage of tissue [7].

Many professionals responsible for tissue banks consider important biological tissue are sterilized by an effective method using ionizing radiation. Sterilization by ionizing radiation is a method that offers advantages over other methods of sterilization, it provides a minimum increase in temperature and leaves no toxic waste, which makes it suitable to be used in addition to being final sterilization [7]. However, some authors concluded that ionizing radiation sterilization can cause structural and biological changes regarding the dose in allografts bones [6,9].

The different types of bone preservation can affect both the maintenance of protein, as the interaction of ionizing radiation with tissue. The different doses of ionizing radiation can also influence bone health, the maintenance of the osteoinductive potential of these grafts. Better knowledge of these parameters can help those responsible for tissue banks in the choice of irradiation conditions and preservation of tissues and quality of the grafts used in transplants.
The objective of this work was to study the effects of the application of gamma radiation and electron beam produced by sources of ^{60}Co and electron accelerator respectively, at doses of 15 kGy, 25 kGy and 50 kGy in demineralized bone tissue preserved frozen and freeze-dried, evaluating possible changes in concentration of total proteins and BMP-2 and BMP-7 to determine the best dose to provide sterility and safety in the preservation of bone graft osteoinductive proteins.

MATERIALS AND METHODS

The selection of samples from five femurs obtained the capture of bone tissue five distinct deceased donors, two male and three female, aged 39-65 years (mean 52 years) according to the Bank of protocol Musculoskeletal tissues of the Santa Casa de Misericórdia de São Paulo.

The diaphyseal femoral was cut out, particulate and the particles with less than 1 mm were used. After this, 48 g of bone tissues (3 g per sample) of each femoral diaphysis were required to produce 16 distinct groups for each donor, totaling 80 samples from 5 donors. Subsequently the samples (42 g) were demineralized in 0,5 N HCL solution in a glass beaker at a ratio of 50 ml of solution per one gram of tissue for 90min at 18±2°C. It was used orbital mechanical stirrer at low speed for constant agitation throughout the demineralization process [4] and subsequently lyophilized (LIO) or frozen (CG).

For the division of groups, demineralized bone tissue (DBM) was divided into two groups: lyophilized or frozen. Samples of groups (DBM) lyophilized and frozen were irradiated at doses of 15, 25 and 50 kGy by two radiation sources: accelerator electrons (AE, electron beam) and Cobalt 60 (γ, gamma rays). Samples were classified as LIO 15, 25, 50 AE; LIO 15, 25, 50 γ; CG 15, 25, 50 AE and CG 15, 25, 50 γ. Two tissue samples non-irradiated control demineralized were created.

Irradiation of samples was performed at the Institute of Energy and Nuclear Research (IPEN). Samples of the groups were irradiated with gamma radiation sources ^{60}Co in the multipurpose irradiator at a dose rate of 1.38 Gy/s or electron beam accelerator with electron industrial dose rate of 3.92 kGy/s. To prevent the variation in temperature, the lyophilized and frozen samples were irradiated at 4ºC and -70ºC, respectively.

For the extraction of total proteins and BMPs bone matrix, guanidine containing buffer was used as described below: was added 0.5 g of tissue from each sample into 5 mL of fresh solution of 4 M guanidine/0.05 M Tris.HCL pH 6.0, containing a mixture of protease inhibitors: 5 mM benzamidine.HCL; 0.1M 6-aminohexanoic acid; 0.5 mM phenylmethylsulfonyl fluoride; 5 mM N-ethylmaleimide, under 4ºC temperature and stirring for a period of 24 hours. Subsequently the tubes were centrifuged at 697 g for 5 minutes. The supernatant was removed and placed in a second tube. Were added again 5 mL of fresh solution of 4 M guanidine/0.05 M Tris.HCL and the mixture of protease inhibitors for the pellet dissolution. The samples were stirred at 4ºC temperature for a period of 5 hours [4]. After that, the samples were centrifuged again and the supernatants added to the previous tube and thereafter fractioned in aliquot and frozen at -80ºC.

The total proteins and BMPs 2 and 7 were quantified by Bradford and ELISA methods, respectively. Measurements were made in triplicate and the result was the arithmetic average. The results for each group were compared to their respective controls as well as the controls were compared. The statistical analysis was performed by comparing the results by one-way ANOVA and followed by the Tukey test for statistics differences ($p < 0.05$).

RESULTS

In Table 1, we can observe the quantification of total protein in mg/g demineralized bone matrix (DBM) and gradual reductions in average concentrations in irradiated groups compared to their respective controls (non irradiated). Statistically significant changes were observed in groups LIO50γ (31,9%), LIO50AE (35,6%), CG25γ (18,3%), CG50γ (39,8%), CG15AE (13,6%), GC25AE (18,3%) and GC50AE (36,1%). BMP-2 and BMP-7 were quantified in order to analyze the effects of ionizing radiation and the possible changes in the

concentrations of these proteins. The results of the concentrations were correlated DBM in grams per gram of total protein.

Table 1: Average concentration decreased total protein percentage in the different study groups compared to the control. (*) Indicates statistically significant difference from the control (p <0,05)

Lyophilized groups	mg/g DBM	Reduction (%)	Frozen groups	mg/g DBM	Reduction (%)
LIO CONTROL	10,70	-----	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 *
LIO 50 γ	7,28	31,9*	CG 50 γ	6,44	39,8 *
LIO 15 AE	9,02	15,7	CG 15 AE	9,24	13,6 *
LIO 25 AE	8,52	20,3	CG 25 AE	8,74	18,3 *
LIO 50 AE	6,89	35,6*	CG 50 AE	6,83	36,1 *

The results obtained in the analyzed groups were recorded in Table 2 and 3, where we observe the average concentration results BMP-2 and BMP-7, in mg/g and ng/g, respectively.

Table 2: Registration of BMP-2 dosages of groups. Reducing the concentration of BMP-2 of irradiated groups in percentage compared to the control (non-irradiated). (*) Indicates statistically significant difference from the control (p <0,05)

Lyophilized groups	BMP- 2 µg/g DBM	(%)	Frozen groups	BMP- 2 µg/g DBM	(%)
LIO CONTROL	2,82	__	CG CONTROL	3,27	__
LIO 15 γ	2,84	0,0	CG 15 γ	2,72	16,8
LIO 25 γ	2,83	0,0	CG 25 γ	2,70	17,4
LIO 50 γ	2,81	0,3	CG 50 γ	2,38	27,2*
LIO 15 AE	2,82	0,0	CG 15 AE	2,67	18,3
LIO 25 AE	2,82	0,0	CG 25 AE	2,66	18,6
LIO 50 AE	1,90	32,6*	CG 50 AE	1,98	39,4*

Table 3: Reduction of concentration of BMP-7 of irradiated groups in percentage compared to the control (non-irradiated). (*) Indicates statistically significant difference from the control (p <0,05)

Lyophilized groups	BMP- 7 µg/g DBM	(%)	Frozen groups	BMP- 7 µg/g DBM	(%)
LIO CONTROL	138,66	__	**CG CONTROL**	148,20	__
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*
LIO 15 AE	101,25	26,9*	**CG 15 AE**	94,20	36,4*
LIO 25 AE	93,78	32,4*	**CG 25 AE**	89,97	39,3*
LIO 50 AE	80,77	41,7*	**CG 50 AE**	70,18	52,6*

The freeze-dried and frozen groups noted the reduction in percentage of average concentrations of BMP-2 and BMP-7 in the different irradiated groups compared to control (non-irradiated). In Table 2, we can see the effects of ionizing radiation on BMP-2 concentrations of the groups studied.

Statistically significant changes with reduction of the average concentrations in relation to the respective control were observed in groups LIO50AE (32.6%), GC50γ (27.2%) and GC50AE (39.4%).

In Table 3, we can see the effects of ionizing radiation on the BMP-7 concentrations in different groups. Statistically significant changes with reduction of the average concentrations of BMP-7 in relation to the respective control were observed in groups LIO15AE (26,9%), LIO25AE (32,4%), LIO50AE (41,7%), CG50γ (44,1%), GC15AE (36,4%), GC25AE (39,3%) and GC50AE (52.6%).

In Figures 1 and 2, we can observe the average concentrations of BMP-2 in relation to the total protein dosed in irradiated and no irradiated groups. There was a statistically significant change in LIO50γ and CG50γ groups compared to the control.

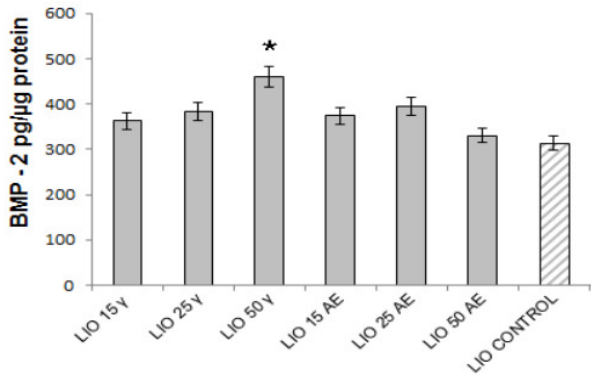

Figure 1: Relationship between the specific BMP-2 concentration and total protein of each lyophilized tissue group non-irradiated and irradiated with gamma radiation and electron beam. (*) Indicates statistically significant difference from the control (p<0,05).

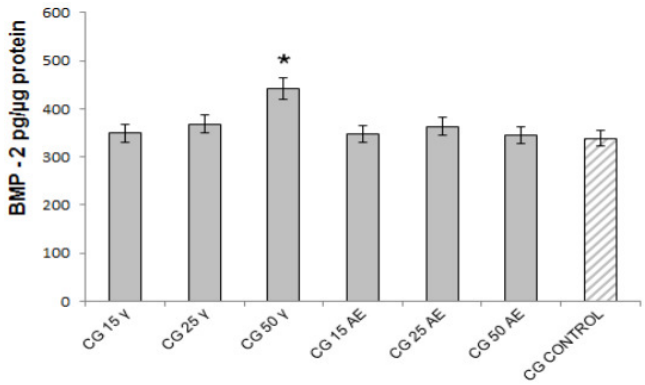

Figure 2: Relationship between specific BMP-2 concentration and total protein of each frozen tissue group non-irradiated and irradiated with gamma radiation and electron beam. (*) Indicates statistically significant difference from the control (p <0,05).

In Figures 3 and 4 we observe the average concentrations of BMP-7 in relation to the total protein dosed in irradiated and no irradiated groups. We noted statistically significant differences in groups LIO50γ, CG15 AE, CG25AE, CG50AE compared to their respective controls.

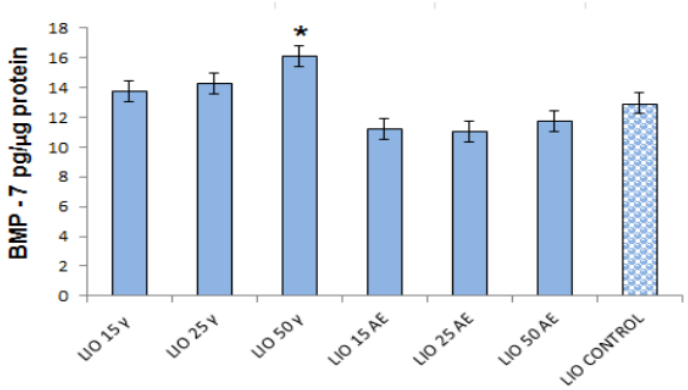

Figure 3: Relationship between the specific concentration of BMP-7 and total protein of each lyophilized tissue group non-irradiated and irradiated with gamma radiation and electron beam. (*) Indicates statistically significant difference from the control (p<0,05).

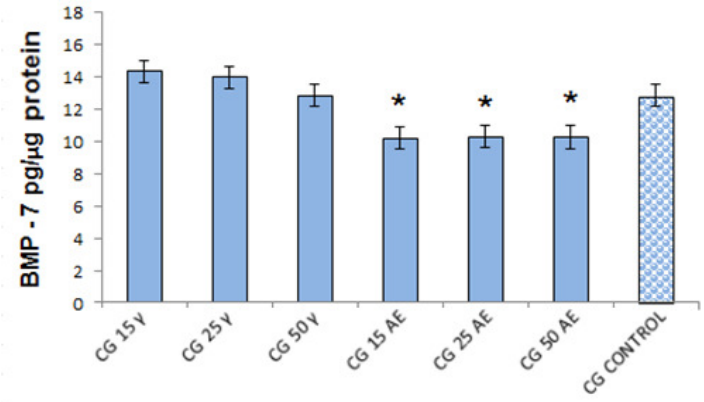

Figure 4: The relationship between the specific concentration of BMP-7 and total protein of each frozen tissue group non-irradiated and irradiated with gamma radiation and electron beam. (*) Indicates statistically significant difference from the control (p<0,05).

DISCUSSION

The increased use of musculoskeletal tissue grafts for reconstructive surgery and the aim of promoting greater safety in transplants and the quality of tissues led Tissue Banks to seek better processing techniques, preservation and sterilization. Gamma radiation is frequently used in tissue banks and proves to be an effective method for providing terminal sterilization of biological tissue, but no reports on the deleterious effects of mechanical and biological properties of the tissue, depending on the radiation dose applied [6].

The application of electron beam radiation is a promising alternative in tissue sterilization process, and it has some advantages compared to gamma radiation, such as fast operating system. The main disadvantage is that the low power of penetration of the electron beam in the material difficult or impossible irradiation, depending on the structure and density of tissues. The activity and the concentration of BMPs are essential for the efficient osteoinductive bone grafts but these proteins can be degraded during the process of sterilization by ionizing radiation and, therefore, may compromise bone transplantation [10].
The protein quantification technique is an important parameter because osteoinduction and osteogenesis are functional processes of bone grafts and depends quantitatively the of proteins presence and bone growth factors, there is a positive correlation between BMPs content in the grafts and the osteoinductivity [5,11].

There are studies with controversies results on the effects of gamma radiation in the osteoinductive potential in demineralized bone grafts. Munting [12] in 1988 concluded that the osteoinduction is totally inhibited by gamma radiation doses above 20 kGy. The studies were conducted with the implementation of DBM in the muscles of mice and rabbits. However, all grafts were irradiated at room temperature what could explain the failures in experiments.

In contrast, other authors, Dziedzic-Goclawska [13] et al in 1991 and Wientroub & Reddi [14] in 1988, irradiated the DBM with gamma rays under controlled temperature conditions using dry ice and promising results have obtained, which reinforces the hypothesis that bone tissue irradiated at low temperatures are less susceptible to the radiation damage. Wientroub & Reddi [14] concluded that a standard dose (25 kGy) of gamma radiation does not alter the osteoinductive potential of DBM implanted in rat muscle and higher doses (30 to 50 kGy) even increased this property.

Likewise, Glowacki [15] in 2005, reported that DBM irradiated with doses of 20 kGy to 40 kGy retained 80% of its osteoinductive activity. Dziedzic-Goclawska in 2002, released the results of frozen bone tissue temperature to -72ºC and irradiated with doses of 35 kGy and 50 kGy, when concluded that were no differences in the osteoinductive potential when compared to non-irradiated bone tissue. However tissues preserved lyophilized and irradiated at room temperature at the same doses were completely absorbed and lost the ability to produce osteoinduction.

With the results of the effects of ionizing radiation in the concentration of osteoinductive proteins, in this study, as reported in Table 2, we see significant reductions BMP-2 in the control (non-irradiated) only in the groups irradiated at doses 50 kGy (LIO50AE, GC50γ and GC50AE). The largest reductions (over 32%) were found in the groups irradiated by electron

beam. At the doses of 15 kGy and 25 kGy, in any of the conditions, we did not see dose dependent changes.

The results of the BMP-7 dose, as presented at Table 3, show significant reductions compared to their respective controls in all irradiated groups by electron beam (LIO15AE, LIOAE25, LIO50AE, CG15AE, CG25AE, CG50AE) and in frozen group irradiated by gamma irradiation at a dose of 50 kGy (CG50 γ). In groups where the reduction was not significant, we observed a tendency to a dose-dependent decrease (7% to 18%).

Comparing the effects of radiation studied in frozen and lyophilized groups, although not statistically significant, frozen groups had greater reduction of the concentration of total protein (13,1% to 36,1%) and BMP-2 (16,8% to 39,4%) and BMP-7 (from 10,1% to 52,6%) when compared to lyophilized groups (12,5% to 35,6%, 0,0% to 32.6%, 7,4% to 41,7%, respectively). This evidence can be explained by higher indirect biological effects of ionizing radiation in the tissues with the greatest presence of water, generating larger amounts of free radicals and therefore possible damage to bone tissues [16].

In Figures 1 and 2, we note a significant increase in average concentration of BMP-2 per gram of protein at a dose of 50 kGy gamma radiation in groups, frozen and lyophilized. In Figure 3 we note a significant increase in average concentration of BMP-7 per gram of protein in the lyophilized group and irradiated at a dose of 50 kGy. In Figure 4 there was a significant reduction in mean concentrations of BMP-7 per gram of protein in the samples of frozen groups and irradiated by electron beam at doses of 15, 25 and 50 kGy. This fact is due to the different effects of ionizing radiation in which specific proteins (BMP-2 or BMP-7) concentrations in relation at the total proteins.

Therefore the effects of ionizing radiation damage to bone graft depend primarily on two factors: (1) the irradiation conditions (radiation dose, dose rate, type of ionizing radiation, radiation temperature); (2) the physical state of the samples, especially the amount of water present in bone tissue [16].

CONCLUSION

The ionizing radiation at high dose (50 kGy) causes significant reductions of 35% to 52% of the total protein concentrations and osteoinductive protein (BMP-2 and BMP-7). It was also observed that the electron beam radiation causes more deleterious effects, in the bone proteins studied, when compared which gamma radiation. However, these conditions are not usually applied to sterilization of bone tissue. In the tissues of the sterilization conditions normally applied to the bone tissue (15 kGy to 25 kGy of gamma rays), reductions in the concentrations of osteoinductive proteins BMP-2 and BMP-7 were lower than 20%.

REFERENCES

1. Associação Brasileira de Transplantes de Órgãos (ABTO). *Registro Brasileiro de Transplantes*. Ano XX, **4**: p.15 (2014).
2. Amatuzzi, M.M.; Croci, A.T.; Giovani, A.M.M.; Santos, L.A.U. "Banco de tecidos: estruturação e normatização". *Revista Brasileira de Ortopedia*, **35 (5)**:p.165-172 (2000).

3. Urist MR, Huo YK, Brownell AG, Hohl WM, Buyske J, Lietze A, et al. “Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography”. *Proc Natl Acad Sci USA*, **81**: 371-475 (1984).
4. Pietrzak, WS, Ali SN, Chitturi D, Jacob M, Woodell-May, JE. “BMP depletion occurs during prolonged acid demineralization of bone: characterization and implications for graft preparation”. *Cell and Tissue Banking*, **12**:81-88 (2011).
5. Blum, B.; Moseley, J.; Miller, L.; Richelsoph, K.; Haggard, W. “Measurement of bone morphogenetic proteins and other growth factors in demineralized bone matrix”. *Orthopedics*, **27**:161–165 (2004).
6. Nguyen, H., Morgan, D.A.F., Forwood, M.R. “Sterilization of allograft bone: effects of gamma irradiation on allograft biology and biomechanics”. *Cell and Tissue Banking*, **8(2)**: 93-105 (2007).
7. Phillips, G. O. Module 5: “Processing. In: Multimedia distance learning package on tissue banking”, 1. ed. Singapura: IAEA (1997).
8. Dziedzic-Goclawska, A. “The application of ionising radiation to sterilise connective tissue allografts”. In: Phillips, G.O. *Radiation and Tissue Banking*, World Scientific (2000).
9. Pekkarinen T, Hietala O, Jamsa T, Jalovaara P. “Gamma irradiation and ethylene oxide in the sterilization of native reindeer bone morphogenetic protein extract”. *Scandinavian Journal of Surgery*, **94**: 67-70 (2005).
10. Puolakkainen, P.A.; Ranchalis, J.E.; Strong, D.M.; Twardzik, D.R. “The effect of sterilization on transforming growth-factor-beta isolated from demineralized human bone”. *Transfusion*, **33**: 679–685 (1993).
11. Ripamonti, U.; Van Den Heever, B.; Sampath, T.K.; Tucker, M.M.; Rueger, D.C.; Reddi, A.H. “Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1 (rhOP-1, bone morphogenetic protein-7)”. *Growth Factors*, **13**: 273–289 (1996).
12. Munting, E.; Wilmart, J.F.; Wijine A.; Hennebert, P.; Delloye, C. “Effect of sterilization on osteoinduction-comparison of 5 methods in demineralized rat bone”. *Acta Orthop Scand,* **59**:34–38 (1988).
13. Dziedzic-Goclawska, A.; Ostrowski, K.; Stachowicz, W.; Michalik, J.; Grzesik, W. “Effect of radiation sterilization on the osteoinductive properties and the rate of remodeling of bone implants preserved by lyophilization and deep-freezing”. *Clin. Orthop. Relat. Res,* **22**; 272:307 (1991).
14. Wientroub, S.; Reddi, H.A. “Influence of irradiation on the osteoindictive potential of demineralized bone matrix calcify”. *Tissue Int.*, **42**: 255– 260 (1988).
15. Glowacki, K.A. “Hemiresection arthroplasty of the distal radioulnar joint”. *Hand Clin*., **21**: 591-601 (2005).
16. Dziedzic-Goclawska, A.; Kaminski, A.; Uhrynowska-Tyszkiewicz, I.; Michalik, J..; Stachowicz, W. “Trends in radiation sterilization of health care products”, International Atomic Energy Agency, Vienna. 231- 256, http://wwwpub.iaea.org/MTCD/publications/PDF/Pub1313_we b.pdf (2008).