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Effects of freezing on bone histological morphology

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Abstract Allograft bone has been widely used for reconstruction of different portions of the skeleton. The fragment of bone harvested must be kept under low temperatures. The cryopreservation also contributes to decrease the antigenic potential of the tissue. Although this technique is considered safe, there is little information about the morphological modifications that the medullary and cortical portions of bone suffer after freezing. Hence, the aim of this study was to investigate the morphology of bone tissue after freezing under different temperatures and periods. Twelve rabbits were used to analyze the effects of two temperatures, −20°C and −70°C, during four periods of time: 30, 60, 90, 120 days. Tissues were analyzed by HE, picro-sirius stains and also by Feulgen's reaction, through qualitative and morphometric ways, considering the area occupied by cells and nuclei, medullary and cortical portions, as well as by collagen expression at cortical. The differences among the treatments were analyzed by Tukeýs test, at 5% significance level. Bone freezing increased cellular and nuclear areas at cancellous bone and diminished nuclear area at the cortical bone. Cortical bone collagen suffered denaturation proportionally to temperature decrease and to freezing duration. These alterations compromised the morphology of tissues after 90 or 120 days of freezing at the temperature of −70°C. Cells necrosed during freezing, contributing to reduce bone antigenicity.

Keywords Bone transplantation · Freezing · Transplantation · Immunology · Necrosis

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Introduction

Bone grafts are widely used for several surgical purposes. Autologous grafts are preferred however, in order to skirt a surgery in a second region, which functions as donor site (Wiltfang et al. 2004; Klongnoi et al. 2006a; Klongnoi et al. 2006b). Frozen allogenous bones are being used as graft (Hou et al. 2005; Jensen et al. 2004). Bone allografts are available in great quantity for large reconstructions and diminish the discomfort to patient when autologous bone is harvested.

Although frozen bone has been definitely considered for grafting procedures (Hou et al. 2005; Jensen et al. 2004; Tetta et al. 2006), few researches have evaluated the effects of low temperature exerts on the histological morphology of bone (Stevenson et al. 1996; Moreau et al. 2000; Blottner et al. 2001; Corcuera et al. 2007; Moreau et al. 2007). Therefore, it is relevant to discuss freezen biology, which developed in parallel to the improving of graft protocols. The aim of this paper was to evaluate the modifications that the morphology of the major components of bone tissue suffered after freezing. Since bone is kept under cryopreservation in the majority of the protocols, two temperatures and four different periods of freezing were evaluated to verify their possible interferences on the alterations of bone morphology.

Materials and methods

All animal experiments were carried out in compliance with the laws and guidelines for experimental use and care of animals, and in accordance with the Ethics Committee for Animal Experimentation of the Bahia Foundation for Science Development, Salvador, Bahia, Brazil.

Four fragments of bone were harvested from the wing of the iliac bone in 12 rabbits. The bone obtained from four animals was prepared to study its original histology, and eight rabbits donated grafts, which were then frozen. Bone harvested from four animals was frozen under −20°C and bone harvested from the others was frozen under −70°C. Bone blocks were kept at each temperature of freezing during 30, 60, 90 or 120 days. Bones from four different rabbits were frozen during each period of time.

Surgery was carried under general anesthesia with the acepromazine, 1 mg/kg, in association with ketamine, 10 mg/kg. After tricotomy and degermation, the skin from the hip was incised. Deep tissue and periosteum dissection exposed the iliac bone. A bone block was harvested by a trephine bur of 1 cm of external diameter. A circular block of bicortical bone, filled with cancellous bone, was harvested. The surgical bed was widely irrigated with 0.9% saline solution. Muscles and the skin were sutured with nylon 4–0. Animals were submitted to euthanasia with excessive administration of ketamine.

After soft tissue removal, each fresh bone blocks were placed inside two special plastic bags, previously sterilized in ethylene oxide, and sent to freezing. A freezer was used to keep the bone under −20°C and another one was used to keep bone under −70°C. Both were located next to the operating room and hence the packed bones harvested were frozen within minutes to hours.

After each freezing period, bone blocks were removed from freezers, immediately fixed in 10% buffered formalin solution and decalcified in tetrahidratated EDTA 1 M, pH of 7,2. Sections of five micrometers thickness were stained with hematoxilina and eosine (HE), picro-sirius and Feulgen's reaction.

The effect of the freezing on the bone histology was analyzed in a qualitative way by an experimenced pathologist, who characterized the tissue morphology as preserved or compromised, according to the features of bone cortical and medullary portions, after freezing. The morphometric evaluation was automatically carried out using the program Motic Image Advance 3,0 in four fields of the medullary portion and four fields of the cortical portion of the bone.

The area of cortical bone matrix, the medullary area, except the spaces of fat accumulation, as well as cells and nuclei area were measured by sections stained with HE. Cortical matrix area, medullary area nuclei area were also measured by sections stained by Feulgen's reaction. Picro-sirius stain aimed to evaluate cortical collagen expression.

Data were analyzed in percentile form. At medullary portion, the percentages of cells and nuclei area were calculated. At cells area, the percentage of the nuclei area was measured. In the cortical portion, the percentage of osteocyte nuclei area was calculated

and the percentage of collagen was estimated in a standard rectangular area of 0.50 mm^2, whose measures were 0.259 × 0.194 mm.

Each percentage was compared taking into account freezing time and temperature, by Analysis of Variance through SPSS 13.0. The statistical difference between each variable was evaluated by Tukeýs test at 5% significance level. Data were presented in function of time and temperature.

Results

Qualitative analysis

Analysis of bone histological morphology performed by an experimental pathologist showed that the tissue preserved its morphology after freezing under −20°C, independently of freezing period. Cellular and nuclear frameworks of bone marrow cells, as well as osteocytes, were also preserved. Freezing under −70°C modified tissue in 25% of the samples after 30 or 60 days. Cryopreservation under this temperature during 90 or 120 days compromised the morphological feature in 100% of samples (Fig. 1). Cells and nuclei experienced, with tissue freezing, an enlargement of its area and their frameworks were poorly defined.

Quantitative analysis

Effect of freezing temperature on cells and nuclei area at medullary portion

Temperature reduction from −20°C to −70°C caused an increase in percentage of the area occupied by cells in the medullary portion, at the first 90 days of freezing. The difference between both temperatures was significant only at the 30th ($P < 0.0001$) and at 90th days ($P < 0.013$). At 120 days, both temperatures caused similar effects on cells area ($P > 0.05$). In all periods and temperatures appreciated, cells area was greater than it was in normal tissue, but no significance was observed at 30 days under the temperature of −20°C or with 120 days, in both temperatures ($P > 0.05$) (Fig. 2).

During any period of observation, reduction of freezing temperature from −20°C to −70°C caused a significant increase in percentage that nuclei represented in cells ($P < 0.001$), even in relation to values surveyed in fresh bone ($P < 0.05$). At the first 30 days, however, temperature of −20°C was not so harmful for nuclei and the increase of their area was not enough to differentiate it from fresh bone pattern ($P > 0.05$) (Fig. 3).

Nuclei area at medullary portion had a similar behavior when freezing temperature reduced from −20°C to −70°C ($P < 0.0001$). HE stain (Figs. 3 and 4) and Feulgen's reaction (Figs. 5 and 6) confirmed each other regarding this finding. The comparison with fresh bone evidenced that temperature of −20°C caused a significant increase in nuclei area in medullary only at 60 days ($P < 0.05$) in sections stained by HE, and at 120 days ($P < 0.05$) in sections stained by Feulgen's reaction. Under −70°C, this increase was significant ($P < 0.05$), independently of freezing period or stain.

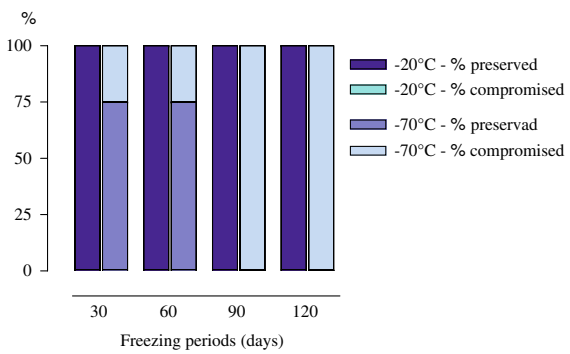

Fig. 1 Qualitative analyses of bone morphology in different days of freezing under −20°C and −70°C, $n = 8$

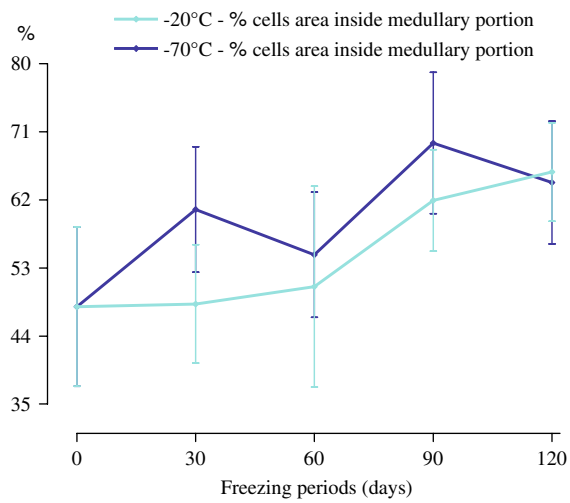

Fig. 2 Percentage of area that cells represented at medullary portion of bone according to the freezing temperature and period, analyzed in sections stained by HE, $n = 12$

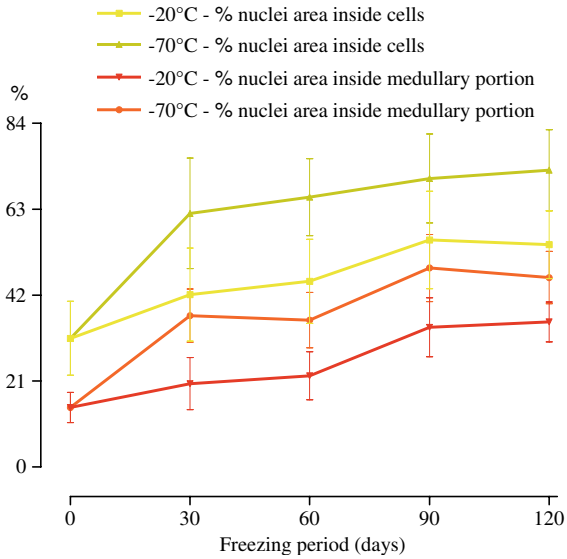

Fig. 3 Percentage of area that nuclei represented in cells and at medullary portion of bone according to the freezing temperature and period, analyzed in sections stained by HE, $n = 12$

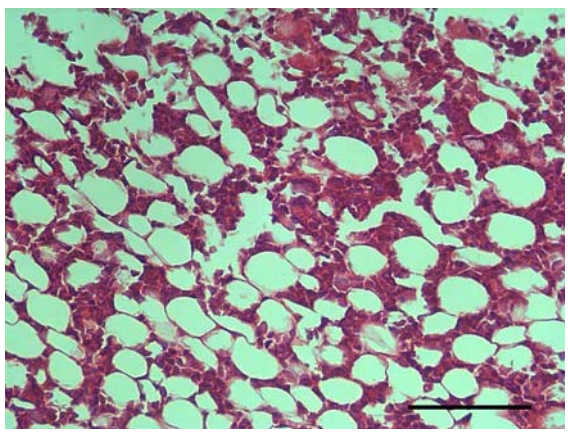

Fig. 4 Aspect of the medullary portion of bone after freezing the iliac bone during 60 days under −70°C. HE, 400×. Scale = 100 μm

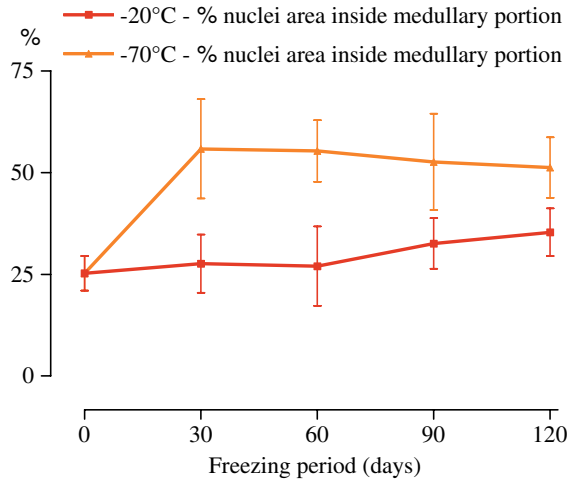

Fig. 5 Percentage of area that nuclei occupied at medullar portion of bone according to the freezing temperature and period, analyzed in sections stained by Feulgen's reaction, $n = 12$

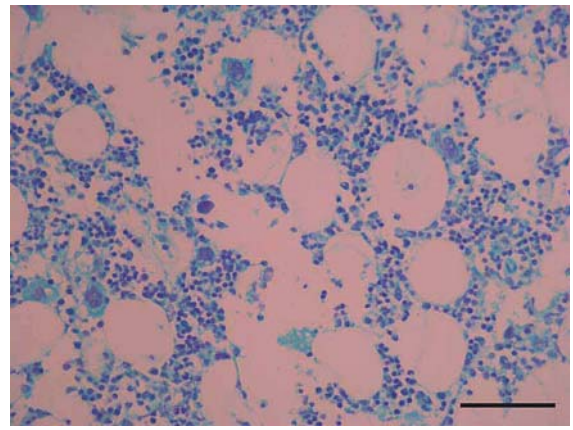

Fig. 6 Aspect of the medullary portion of bone after freezing the iliac bone during 60 days under −70°C. Feulgen's reaction, 400×. Scale = 100 μm

Effect of freezing period on cells and nuclei area at medullary portion

Under −20°C, as freezing time drew out, the area that cells occupied in medullary region increased. The increase was significant only when 30 or 60 days were compared to 90 or 120 days ($P < 0.05$). Under −70°C, a great variation in cells area was observed throughout the studied periods. Difference was significant when results of 30 days were compared with those of 90 ($P < 0.005$), and results of 60 days were compared with those of 90 days or 120 days ($P < 0.002$) (Fig. 2).

Nuclei area in cells, when tissue was frozen under −20°C, increased in function of time but it was only significant when 30 or 60 days was compared to 90 or 120 days ($P < 0.019$). Under −70°C, area of nuclei in cells experimented a gradual increase, but it was only statistical relevant when 30 days were compared to 90 ($P < 0.033$) or 120 days ($P < 0.009$) (Fig. 3).

The increase in the percentage that nuclei occupied in medullary portion of iliac bone was gradual but it was only significant when 30 or 60 day were compared to the last two periods of freezing ($P < 0.0001$) in any temperature studied on the sections stained with HE (Fig. 3). On Feulgen's reaction, a gradual increase was observed in nuclei area at medullary portion after freezing under −20°C, but differences between the periods of observation were only significant when the 30th day was compared to the 120th day ($P < 0.005$), and 60th day was compared to the 90th ($P < 0.038$) or 120th day ($P < 0.002$). Under −70°C, the increase of nuclei in medullary portion on 30th day was important in relation to the value surveyed in fresh bone ($P < 0.05$) and suffered little modification according to elapsing of time ($P < 0.516$) (Fig. 5).

Effect of freezing temperature on osteocytes nuclei area

At sections stained by HE, area of osteocytes nuclei presented reduction when values were compared to the fresh bone, in any temperature and in any day ($P < 0.05$). In the 30th day, temperature reduction from −20°C to −70°C caused a higher decrease in this area ($P < 0.022$). After 60 days, both temperatures were equivalent ($P < 0.259$) (Fig. 7).

At sections stained by Feulgen's reaction, freezing under −20°C caused a reduction in osteocytes nuclei area, in relation to fresh bone, in the 30th, 60th and 120th days ($P < 0.05$). Under −70°C, the reduction, in relation to fresh bone, was evidenced in any day, but was only significant in the 30th and 120th days (Fig. 8). Temperature of −20°C was different from temperature of −70°C only at the 90th day, due to an increase observed in nuclei area at the higher temperature ($P < 0.05$) (Fig. 7).

Effect of freezing period on osteocytes nuclei area

At sections stained by HE, temperature of −20°C caused a gradual reduction in the percentage that osteocytes nuclei occupied in the cortical portion of the bone. Between the 30th and the 60th day of freezing, decrease was significant ($P < 0.05$). These two periods presented lower values than those of fresh bone ($P < 0.018$). Area of the nuclei was not statistically different between 60, 90 or 120 days

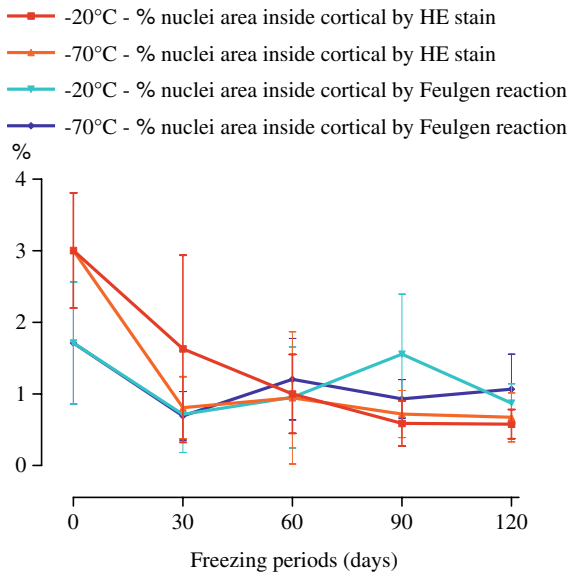

Fig. 7 Percentage of area that osteocytes nuclei represented at cortical tissue according to the freezing temperature and period, analyzed in sections stained by HE and Feulgen's reaction, $n = 12$

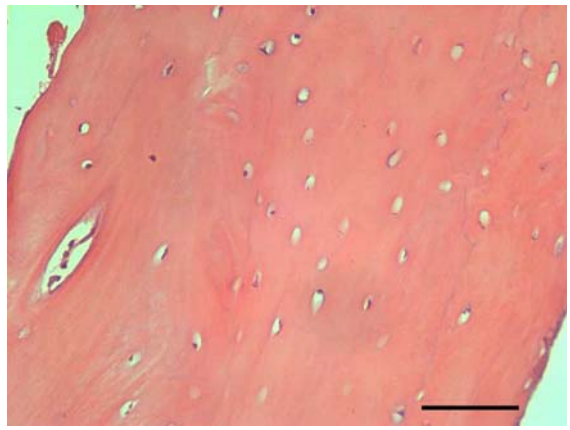

Fig. 8 Aspect of the cortical portion after freezing the iliac bone during 30 days under −20°C. HE, 400X. Scale = 100 μm

($P < 0.117$). Under temperature of −70°C, the most important reduction in osteocytes nuclei area occurred until 30 days of freezing and kept the same pattern at other days of observation ($P < 0.190$) (Fig. 7).

Feulgen's reaction evidenced that freezing under −20°C during 30 days promoted a reduction at osteocytes nuclei area when compared to fresh bone ($P < 0.05$). At 90 days, osteocytes nuclei area increased, becoming different from the one at other

periods ($P < 0.008$) and resembling the fresh bone area ($P > 0.05$). Under −70°C, the reduction that occurred until the 30th day differed only from that observed at 60 ($P < 0.001$) or 120 days ($P < 0.017$), whose values were similar to the values measured at the fresh bone (Fig. 7).

Effect of freezing temperature on collagen area at the cortical portion of the bone

Any reduction of temperature, independently of how low the freezing time was, caused alterations in collagen that was enough to differentiate from the normal pattern ($P < 0.0001$). Collagen area measured at −70°C, was lower than the area measured at −20°C in the first three periods of time ($P < 0.005$). At 120 days, the reduction caused by temperature of −20°C was great enough to equalize it to the lower temperature ($P < 0.909$) (Fig. 9).

Effect of freezing period on collagen area at the cortical portion of the bone

Freezing under −20°C during 120 days caused a reduction of collagen area at cortical portion of bone, which differentiated it from the area measured in any other period of freezing ($P < 0.001$). Cryopreservation during 60 days caused a discrete increase in this area, which was enough to differentiate it from all other studied days ($P < 0.045$). Collagen area did not significantly differ between the 30th and 90th day ($P < 0.177$) (Fig. 9).

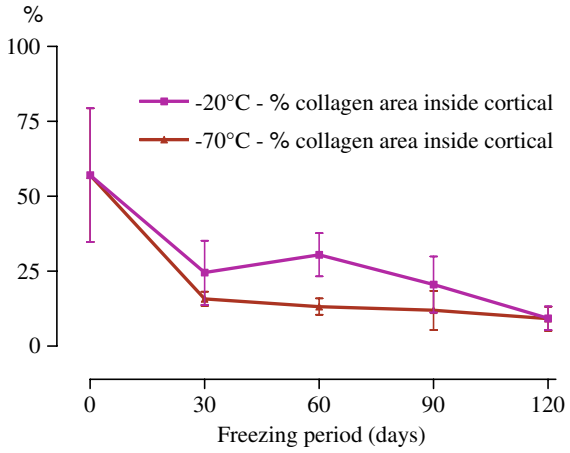

Fig. 9 Percentage of area that the collagen represented at the cortical tissue according to the freezing temperature and period, analyzed in sections stained by picro-sirius, $n = 12$

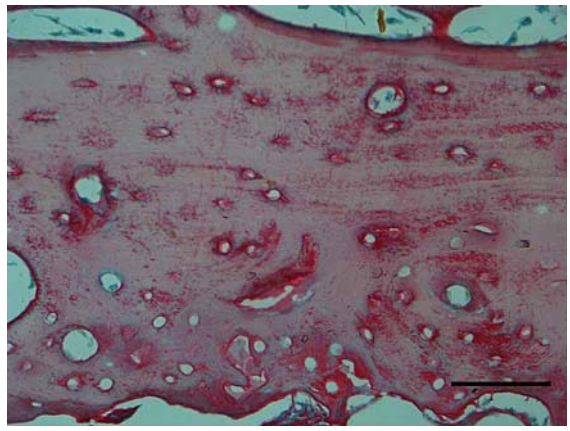

Fig. 10 Collagen aspect at cortical after freezing the iliac bone during 120 days under −20°C. Picro-sirius, 400×. Scale = 100 μm

At temperature of −70°C, a difference of 30 days between the periods of freezing did not cause alterations at collagen area. It only differed when freezing period of 30 days was compared to 90 ($P < 0.012$) or 120 days ($P < 0.001$), or when 60 days was compared to 120 days ($P < 0.009$) (Fig. 10).

Difference between HE stain and Feulgen's reaction

When the percentage of nuclei at medullary portion of bone was evaluated, the difference between Feulgen's reaction and HE stain was only significantly different when the fresh bone was analyzed and the tissue frozen under −70°C ($P < 0.05$). At this temperature, the measures performed at HE stain exhibited higher values. In relation to freezing time, differences between the stains were statistically significant at period of 30 and 60 days ($P < 0.005$).

Regarding the percentage of nuclei at cortical, differences between these stains were significant for fresh bone, temperature of −70°C and for the periods of 30, 60 and 90 days ($P < 0.005$). Despite this significance, at cortical, the differences between the two stains were mild and Feulgen's reaction presented the higher values.

Discussion

Freezing is the most common method for bone storage until its use for skeletal reconstructions. Protocols

suggest that bone must be kept at low temperatures for a period higher than 90 days and could be stored for up to five years (Farrington et al. 1998).

Qualitative analysis concluded from the impressions of an experimental pathologist showed that freezing modified cells and nuclei size, which coarsely modified the aspect of tissue after 90 or 120 days of freezing under −70°C. The measures of nuclei and the cells area evidenced more alterations than qualitative evaluation, probably because the automatic measurements identified mild alterations that had become unnoticeable to human eye.

It is worthy to highlight that the morphometric differences can be statistically significant, but they may not express proportional biological impact. Bone morphology that was classified as poorly preserved, corresponded to those in which the morphometric alterations were also more accentuated. The bone blocks that showed this pattern presented a severe disarrangement at medullary portion, which is the major factor for frozen bone graft immunogenicity reduction (Lee et al. 1997). Thus, it seemed coherent that the qualitative evaluation was efficient in identifying bone blocks that, after frozen, were adequate for being grafted.

Freezing caused expansion in the area occupied by cells and its nuclei. Water contained at citosol and nucleus experienced a thermal dilatation with temperature reduction that resulted in the expansion and rupture of cellular and nuclear membranes (Corcuera et al. 2007). Other researchers also evidenced that spermatozoa cryopreservation causes ruptures of its membrane at the acrossomial region (Blottner et al. 2001). The action of low temperatures on cells has justified the utilization of liquid nitrogen cryotherapy as a method to assure adequate surgical edge after odontogenic tumors curettage (Reis et al. 2006a).

Temperature reduction to −20°C during 60 days, caused alterations on cellular framework that were similar to the ones verified at −70°C. Nuclei were even more sensible to temperature reduction and the membrane expansion of this important organelle was evident since the first period of freezing.

Cells at cortical were submitted to the same mechanism during freezing. However, osteocytes nuclei area was smaller than fresh bone ones, independent of freezing period. No significant difference was observed between different temperatures. This probably occurred as consequence of osteocytes disappearance in some gaps during freezing, as also occurred during other treatments used to induce bone antigenicity reduction (Dumas et al. 2006; Kawalec-Carroll et al. 2007).

Osteocytes disappearance masked the volume expansion experienced individually by cells, resultant from water dilatation during freezing. The necrosis of these cells should not be understood as an undesired phenomenon, since the major function of bone cortical is to provide a framework for new bone formation and, besides, to assure graft mechanical strength (Tshamala et al. 1999).

Collagen present at cortical was, undoubtfully, the most sensible bone component to freezing. Temperature reduction to −20°C caused important alterations on the expression of this protein in the tissue, which was significantly aggravated by freezing under −70°C. Under each temperature, alterations were aggravated after 60 days of freezing.

Freezing is also harmful for extracellular matrix and this effect had also been imputed to icing formation within tissues (Laouar et al. 2007). Low temperatures used for cartilage cryopreservation diminished collagen expression, as also did with other glycoproteins, such as chondroitin sulfate (Laouar et al. 2007; Moreno and Forriol 2002). Treatment with hydrogen peroxide also promoted analogous alterations. After these treatments, collagen fibers suffered ruptures with intense fibrils formation. Fibrils were exposed and disposed in a disorganized manner, worsening cortical relief (Dumas et al. 2006). Alterations found through the examination of cortical ultrastructure by scanning electron microscopy (Dumas et al. 2006) were analogous to the ones found by picro-sirius stain at the present research.

It is well demonstrated that type II collagen, isolated, induced high titles of IL2, IL4, IFN-gama and specific IgG and IgM (Kawalec-Carroll et al. 2007). Certainly due to the alterations that this protein exhibits after treatment, specific imunoglobulins against type II collagen had not been detected in the serum of rats that received frozen bone or bone submitted the photo-oxidation. These speculations also justify the delay on the healing of experimental cavities grafted only with isolated type II collagen (Schlegel et al. 2004).

Feulgen's reaction was adopted at this work, considering that it would be a more efficient marker of nuclear ratios after different freezing temperatures

exposure. The results of this stain showed that the diameter of this organelle experienced little variation throughout the freezing period and under both temperatures.

The major characteristic of Feulgen's reaction is a high sensitivity to stain cellular DNA and it is frequently used to evaluate chromosomal alterations (Reis et al. 2002; Reis et al. 2006b). The linearity of the values observed for nuclei area at this stain suggests that DNA would suffer the impact of temperature reduction on a lower intensity than the nucleus as a whole. Other researches that studied cartilage cryopreservation (Laouar et al. 2007) and spermatozoa cryopreservation (Blottner et al. 2001), also found that the DNA remained preserved after the treatments employed.

DNA is a highly condensed structure, due to its noble role for cell and for evolution overall. This condensation occurs around the histones forming the nucleossome. Thermal dilatation imposed by water, had little influence on this characteristic of the desoxiribonucleic acid. Under −70°C, the proteins responsible for DNA condensation suffered a more evident denaturation, causing chromatin dispersion, as observed by Feulgen's reaction, and in agreement to other survey (Corcuera et al. 2007). The diameter increasing of the complex formed by the chromatin and the histones with temperature reduction have been previously comproved (Horowitz et al. 1990).

HE stain and Feulgen's reaction presented different values for the majority of the studied samples. Other works also compared Feulgen's reaction with other techniques to evaluate the incidence of chromosomal alterations in spermatozoa (Sprecher and Coe 1996; Beletti and Mello 2004). In agreement with the present paper, the findings obtained with this special stain differed from those observed in other evaluation protocols.

In conclusion, morphological alterations observed in this experiment were cells and nuclei area enlargement, osteocytes disappearance and collagen disorganization. It can also be stated that the temperature of −70°C was more effective than the temperature of −20° in necrose bone cells. Perhaps, a longer time of freezing, under −20°C, may achieve similar results, however it may not be a safe protocol to reduce immunogenicity. Tissue necrosis under this treatment is strongly associated to ice formation inside the cell and extracellular matrix.

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