Effect of different cryosurgical protocols using liquid nitrogen on bone tissue: a histomorphological analyze

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Abstract

The aim of the present experimental study was to evaluate the morphological effects of different liquid nitrogen cryosurgery protocols on bone tissue. The femoral diaphyses of 42 Wistar rats were exposed to three local and sequential applications of liquid nitrogen for 1 or 2 min, intercalated with periods of 5 min of passive thawing. The animals were sacrificed after 1, 2, 4 and 12 weeks and the specimens obtained were processed and analyzed histomorphologically. Histologically, an increase in bone necrosis was observed for the two protocols in the second week after cryotherapy. A significant osteogenic phase was observed after 4 weeks. Moreover, complete remodeling process was encountered at the end of the morphological observation period (12 weeks), especially on one-minute protocol. Thus, this study indicated that the 2-min protocol produced more marked bone necrosis than the 1-min protocol. In addition, the second experimental week was critical for bone necrosis with either cryotherapy protocol. Further studies are important for the understanding of the long-term behavior of bone tissue after cryoapplication of liquid nitrogen.

Key Words: *cryosurgery; jaw; animal models.*

Introduction

Cryosurgery, a method that uses low temperatures to produce local tissue destruction (1), became a well-established technique in the 1970s and 1980s (2) and is widely used for the treatment of various oral soft tissue lesions because of its simplicity, easy handling and therapeutic efficacy (3).

The maxillofacial region can be affected by a variety of locally aggressive benign lesions that are characterized by a high rate of recurrence after conservative surgical treatment (4). On the other hand, radical treatment of these conditions often results in severe functional and esthetic impairment for the patient (5). Within this context, cryotherapy has been shown to be an important technique, mainly because of the simplicity of the cryosurgical procedure and because it preserves a non-pathological bone remnant that can be devitalized without the need for surgical resection and may also serve as a recipient site for bone grafts (6).

Various cryosurgery protocols are used in clinical practice, but the morphological alterations resulting from these procedures have not been well investigated and are not completely understood. In addition, standardized experimental studies using rats as an animal model and comparing morphologically the therapeutic protocols used are scarce in the literature. Therefore, in view of the impracticability of human studies and the need for a better understanding of the long-term morphological alterations produced by cryotherapy, the objective of the present study was to analyze histomorphologically the effects of different protocols of liquid nitrogen application on bone tissue.

Material and Methods

Animals

Forty-two male Wistar rats aged 16 weeks (360-460 g), randomly chosen from the Central Animal House of the Federal University of Ceará, Fortaleza, Brazil, were used in this study. The animals were housed in separate cages on a 12-24 h light/dark cycle at 23-25°C, with free access to food and water. The experiments were reviewed and approved by the Animal Care and Use Committee of the same institution (protocol number 09/06) and were conducted according to recommended guidelines on animal experimentation.

Cryosurgery protocols

Freezing was carried out in a CRY-AC®-3 cryostat, model B-700, (Brymill®, imported from CRY-AC®, Osaco-SP, Brazil) using liquid nitrogen as coolant and a closed cryoprobe with a flat surface measuring 1 mm in diameter. The animals were divided into two groups according to the cryosurgery protocol used: group A was submitted to three 1-min freeze cycles intercalated with periods of 5 min of passive thawing, for a total freeze time of 3 min; group B was submitted to three 2-min freeze cycles intercalated with periods of 5 min of passive thawing, for a total freeze time of 6 min.

Surgical procedures

The rats were anesthetized by intraperitoneal injection of 2.5% tribromoethanol (0.1 mg/100 g body weight) (Sigma®, St. Louis, MO, USA). Next, a 1.5-cm linear incision was made along the lateral aspect of the right thigh from the proximal femur. The anterior thigh muscles were cut longitudinally and the femoral diaphysis was exposed. The joint ligaments were released and the tip of the closed probe was positioned on the bone surface 1 cm from the head of the femur. Retractors were carefully positioned to avoid damage to adjacent soft tissues during the freezing process. After cryosurgery, the muscles were returned to their initial position, and the soft tissues and skin were sutured. The animals were allowed to recover and were placed in individual cages. No drugs were administered postoperatively.

Histologic preparation

Groups of rats were sacrificed by cervical dislocation 1, 2, 4 and 12 weeks after surgery. The right femurs were removed, fixed by immersion in 10% neutral buffered formaldehyde (Sigma®, St. Louis, MO, USA) for 48 hours at room temperature, and then decalcified in 5% acid nitric (Sigma®, St. Louis, MO, USA) for 10 days. After decalcification, the specimens were dehydrated in a graded ethanol series, placed in xylene (Vetec®, Rio de Janeiro, Brazil), and embedded in paraffin (Vetec®, Rio de Janeiro, Brazil) in such a way that the femur could be sectioned in the sagittal plane. The site of cryoapplication was determined by positioning a millimeter rule 1 cm from the femoral head. Sections (4 μm thick) were cut sequentially from the lateral border to the site of cryoapplication and stained with hematoxylin and eosin (Merck®, Rio de Janeiro, Brazil).

Histomorphological analysis

A double-blind quantitative descriptive morphological study was conducted to determine the alterations produced in cortical bone. The observers were unaware to which animal group the slide belonged, which was previously codified. Two previously calibrated examiners performed the analysis.

Results

Control group

Descriptive analysis of this group showed a normal bone matrix containing osteocytes of different sizes that were trapped in bone lacunae. The cells presented basophilic round or flattened nuclei and were arranged among the vascular canals of the haversian system (Fig.1).

One-minute protocol

• 1 week

A small area of necrosis was noted in cortical bone, which could be clearly distinguished from normal bone tissue. In addition, few histological fields showed empty
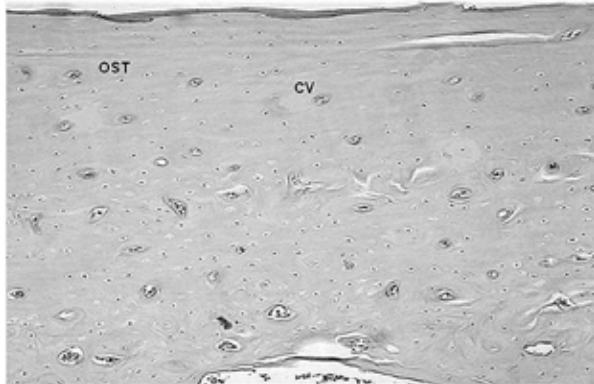
Fig. 1. Control group showing bone matrix containing normal osteocytes (OST) and harvesian vascular canals (CV). H-E, x100.

irregular formation of new bone amidst a population of osteocytes was noted in the bone matrix, an event characterizing the process of bone remodeling (Fig. 3A).

• 12 weeks

A small superficial band of bone necrosis in both extent and depth remained in cortical bone 12 weeks after treatment. Few empty lacunae were observed and necrotized vascular canals were rare. In contrast, marked deposition of new bone in a more compact bone matrix was noted (Fig. 3B). No alterations were observed in the contralateral cortical bone.
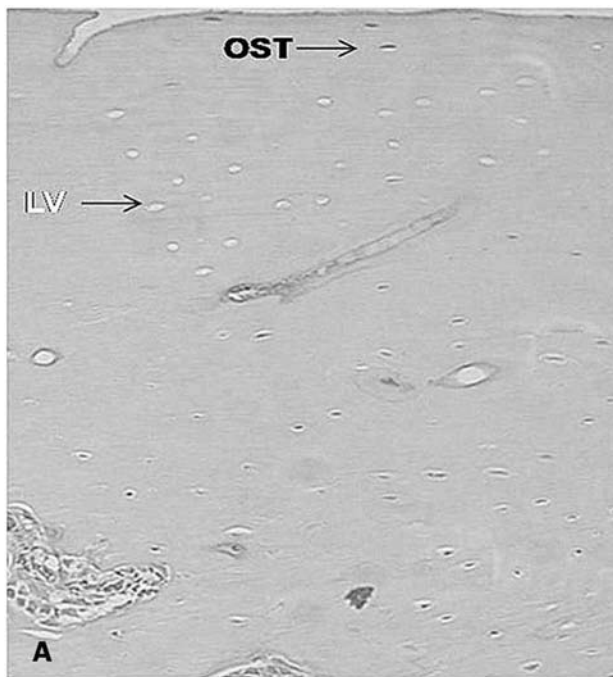
Two-minute protocol

• 1 week

Fig. 2. Necrotic phase with empty osteocytes (LV) after 1 week. One-minute group (A); Two-minute group (B). H-E, x100.

nutrient canals amidst bone lacunae devoid of osteocytes (Fig. 2A). The contralateral cortical bone showed no alterations.

• 2 weeks

An increase in the depth and extent of bone necrosis was observed, but the necrotic area did not reach the whole cortical bone depth. The lack of osteocytes resulted in the formation of empty lacunae, in addition to necrotic nutrient canals in the areas affected (Fig. 2B). No signs indicative of bone necrosis were observed in cortical bone contralateral to the site of treatment. In addition, normal tissue characterized by preserved cellularity and vascularity was observed in most histological fields.

• 4 weeks

A reduction in the depth of bone necrosis characterized by a superficial band of empty bone lacunae and rare degenerated vascular canals was observed. In contrast,

Substantial cortical bone necrosis in terms of depth and extent was observed, although few fields presented complete necrosis of the area analyzed, which was characterized by the death of osteocytes, including the region bordering medullary tissue. Vascularization was also altered as indicated by the presence of various degenerated nutrient canals (Fig. 4A). No necrosis was observed in cortical bone contralateral to the site of treatment.

• 2 weeks

Peak cortical bone necrosis was observed between groups after 2 weeks, which was characterized by the marked formation of empty lacunae in most histological fields analyzed and the presence of various degenerated vascular canals. The areas representative of this group showed deep necrosis, completely involving the cortical bone (Fig. 4B). However, the cortical bone on the con-
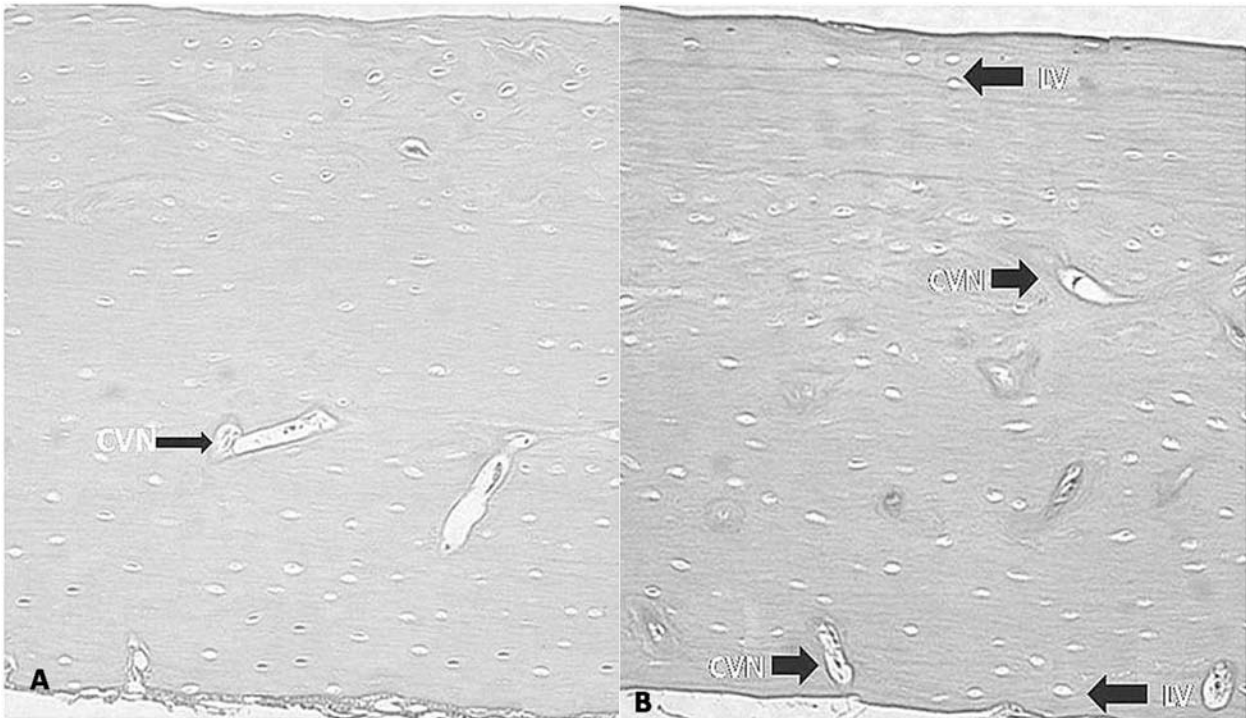
Fig. 3. *Necrotic phase with empty osteocytes (LV) and harvesian vascular canals (CVN) after 2 week. One-minute group (A); Two-minute group (B). H-E, x100.*
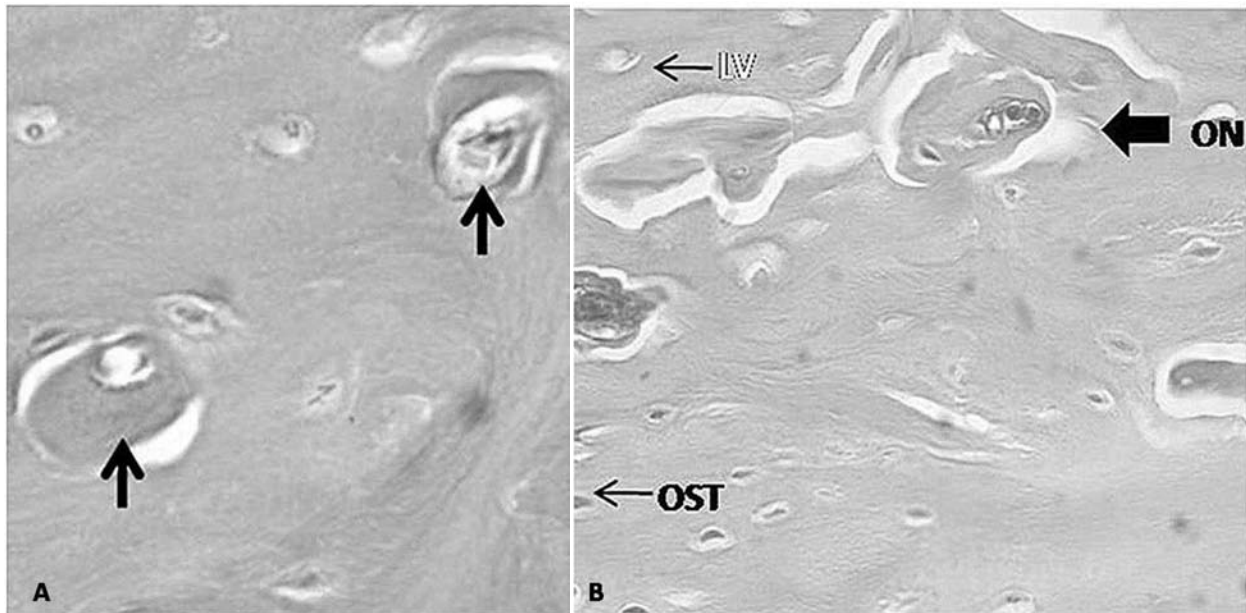

Fig. 4. *Osteogenic phase with normal osteocytes (OST) and new bone (ON) after 4 weeks. One-minute group (A); Two-minute group (B) showing complete cortical necrosis. H-E, x400.*

tralateral side remained intact.

• 4 weeks

After 4 weeks, a reduction in the depth of cortical bone necrosis was observed at the site where the cryogen was applied. A band of bone lacunae without osteocytes was observed, in addition to few necrotized nutrient canals (Fig. 5A).

• 12 weeks

A marked reduction in the area of cortical bone necro-sis was observed 12 weeks after treatment in the groups submitted to the two-minute protocol, which was charac-terized by a more superficial band of necrotic tissue of small extent. Similarly, the process of bone remodeling was significant in all treated animals, with the observa-tion of various areas where mineralized material was forming. Vascularization was completely reestablished, with the observation of areas containing various nutrient canals (Fig. 5B).
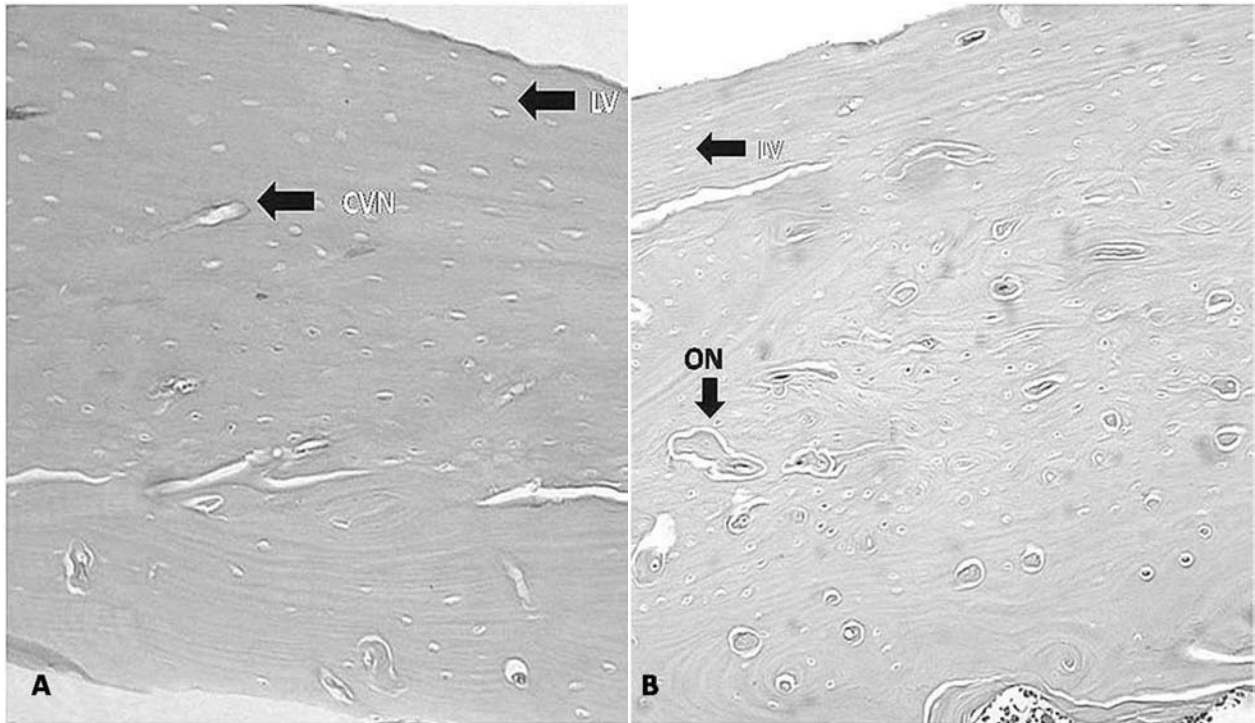
Fig. 5. *Remodeling phase with superficial necrotic area (LV; CVN) after 12 weeks. One-minute group (A); Two-minute group (B) showing abundant presence of new bone (ON). H-E, x100.*

Discussion

The maxillomandibular complex is affected by a variety of benign lesions that often present an aggressive biological behavior and a high potential of recurrence, such as ameloblastomas (7,8), keratocystic odontogenic tumours (9), odontogenic myxomas (10), and central giant cell lesions (11). The ideal management of these conditions would be an approach that permits complete resection of the lesion and, at the same time, the least functional and esthetic impairment for the patient. The effectiveness of cryosurgery is reflected in the low rates of recurrence reported by different investigators (5,12).

The process of freezing tissue for devitalization has changed little in the recent years. Fast freezing, slow thawing, and repetition of the freeze-thaw cycle are the essential tenets of cryosurgery. These facets of technique are well known to be destructive to cells. The mechanisms of injury are complex. Direct cell injury is caused by crystallization of water, with its attendant deleterious effects. Recently, apoptosis has been recognized as a mode of cell death. Additionally, the malfunction of microcirculation in thawed tissue is a long-recognized part of cryogenic injury to tissue (1).

The use of animal models in dental studies has permitted the analysis of aspects of bone architecture that are difficult to investigate in humans. Rats are commonly used because they present some advantages over other animals. In addition, according to Najjar and Kahn (13), healing and remodeling in the mandible are similar to those observed in the femur and tibia. Despite different embryologic origins, the similarity in the healing process is due to the physiological forces that act on these bones.

Regardless of the time of application, three freezing cycles were standardized since animal studies have shown increased cell damage when the tissue is submitted to a state of cooling at temperatures that permit the occurrence of the recrystallization phenomenon. In addition, the repetition of freezing cycles exerts a more significant tissue effect since the cells are subjected to more harmful and cumulative alterations, including physical and/or chemical damage. Thus, the deleterious effects on cells and their constituents in the first freezing cycle might be responsible for an increase of thermal transmission in subsequent cycles (14).

Histologically, a necrotic phase was observed as early as in the first week after cryogen application, a finding also reported in studies on rat mandibles conducted by Bradley and Fisher (15) and Bradley (16). In agreement with these authors, in the present study a superficial band of osteocyte loss, with the consequent formation of empty lacunae, was identified after the application of either protocol. In contrast, Popken et al. (17) observed total cortical necrosis in the first week, although the authors adopted a protocol of 15-min applications to femoral sheep bone. Also in contrast to the present findings, Emmings et al. (18) demonstrated complete necrosis of cortical bone within the first 7 days.

In the second week after cryotherapy, an increase in bone necrosis was observed for the two protocols. Parti-

cularly the groups submitted to the two-minute protocol showed complete necrosis of cortical bone in various histological fields analyzed. Similarly, Kuylenstierna et al. (19) observed complete devitalization of mandibular cortical bone in rabbits 2 weeks after the application of liquid nitrogen for 3 minutes each. However, in contrast to these authors, no areas of deposition of new cortical bone were observed after the application of either cryotherapy protocol.

A significant osteogenic phase was observed after 4 weeks, which was characterized by a reduction in the number of lacunae devoid of osteocytes and an increase in the number of osteocytes and intact vascular canals when compared to the second week. In addition, the femoral cortex presented areas of deposition of newly formed bone, which was more pronounced for the two-minute protocol. These findings agree with the experiments of Bradley (16) and Keijser et al. (20).

The smallest number of necrotic cells was observed in the groups analyzed at the end of the morphological observation period (12 weeks), especially those submitted to the one-minute protocol, in which a small band of superficial empty lacunae could be noted. In agreement with the microscopic findings of Bradley and Fisher (15), in both protocols the cortical bone consisted of a richly cellular and vascularized matrix, with this week corresponding to the period of maximum vascularization. In addition, the osteogenic phase was more consistent, especially in the case of the two-minute protocol, with the observation of the formation of lamellar and mature bone in various areas. Similar findings have been reported by Keijser et al. (20).

Data derived from experimental studies are important for the understanding of the mechanisms whereby cryosurgery exerts its effects. Therefore, although the present results cannot be readily extrapolated to clinical practice, they are important for the understanding of the long-term behavior of bone tissue submitted to distinct cycles of liquid nitrogen application and may serve as a basis for future clinical studies or even contribute to already existing clinical protocols.

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