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Bacteriological evaluation of bone grafts stored by cryogenic freezing at –24°C from a canine (*Canis familiaris*) bone bank

Avaliação bacteriológica de enxertos ósseos submetidos à técnica criogênica a –24°C em banco de ossos de cães (*Canis familiaris*)

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Abstract

The scientific basis of bone transplantation was established during the mid-nineteenth century, when the osteogenic properties of the bone and periosteum, beneficial influence of cold preservation of bones, and first conventional attempt to store bones for elective use, were described. A bone bank has several advantages, such as the immediate availability of grafts in large quantities, and different shapes and sizes, and maintenance of osteoinductive activity in grafts. In addition, it reduces patient blood loss, surgical time, and quantity of anesthetics required. Clinical applications include the correction of comminuted fractures, treatment of non-union bones, and replacement of bone loss due to infections or malignancies. The success of these procedures depends on the preservation and integrity of the graft. This study aimed to evaluate the feasibility of maintaining a canine bone bank (*Canis familiaris*) by quantifying the bacterial contamination of bone grafts preserved at –24°C. The samples were evaluated through a monthly assessment of bacteriological cultures over a period of 6 months. The harvest method was efficient and sterile, reducing the risk of contamination. We conclude that the techniques chosen for the implementation of a bone bank were effective and feasible (use of a common freezer reaching a temperature below –24°C). The viability of the bones was attested during a 6-month period, and the samples demonstrated a 100% sterility rate.

Key words: Bone bank, canine, cryogenics, grafts

Resumo

A base científica do transplante ósseo foi estabelecida na metade do século IX quando foram descritas as propriedades osteogênicas do osso e do periósteo, a influência benéfica do frio na preservação das mesmas, e a primeira tentativa convencional para armazenar ossos para uso eletivo. As vantagens do banco de ossos são a disponibilidade imediata do enxerto em quantidade, forma e tamanhos variados, com a manutenção de sua atividade osteoindutora, e redução do tempo cirúrgico, anestésico e perda sanguínea do paciente. Dentre as aplicações clínicas cita-se a correção de fraturas cominutivas, tratamento de não união óssea e reposição de perda óssea devido a infecções ou neoplasias. O sucesso destes procedimentos depende dos parâmetros de conservação e integridade do enxerto. Este estudo teve como objetivo avaliar a viabilidade da manutenção de banco de ossos de cães (Canis familiaris) quantificando a contaminação bacteriológica dos enxertos ósseos, submetidos à técnica criogênica (-24°C) e avaliados através de realização mensal de culturas bacteriológicas por um período de seis meses. O método de coleta mostrou-se eficiente e propiciou adequada manutenção de esterilidade

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durante o período de coleta, diminuindo o risco de contaminação dos ossos. Concluímos que as técnicas escolhidas para implantação do banco de ossos mostraram-se eficientes e factíveis, com freezer comum de temperatura abaixo de –24°C, comprovou-se a viabilidade dos ossos por um período de 6 meses, obtendo-se taxa de esterilidade dos mesmos de 100% através das culturas bacteriológicas.
Palavras-chave: Banco de ossos, cães, criogenia, enxertos

Introduction

In 1867, Ollier established the scientific basis of bone implants through the study of the osteogenic properties of the bone and periosteum. During the twentieth century, Inclan (1942) and Wilson (1947) described the use of preserved bone in orthopedic surgery, and Friedlander (1985) reported the beneficial effects of cold preservation on properties of the bone and periosteum.

Bone defects, resulting from trauma, postoperative complications, and mutilating surgeries associated with the removal of tumors, are routine in veterinary hospitals and clinics. Bone grafts, and all their variants, are the major viable option for the structural repair of substantial bone loss. The search for alternatives to correct these issues has currently led many researchers to join this line of study (SALBEGO, 2010).

The creation of a bone bank enables the provision of biological material for a number of orthopedic procedures. In fact, the growing need for musculoskeletal tissue for transplantation is reported to be due to the development of new surgical techniques. This need has prompted many researchers and organizations to develop their own source of tissue for transplantation (ALENCAR; VIEIRA, 2010).

Different bone banks use different methods of preservation. However, the effects of these methods on the quality of the graft have not been completely elucidated. These methods must be simple, inexpensive, non-toxic, and sterile. In addition, they must effectively preserve the mechanical and biological properties of the bone, and provide safe tissues, which are compliant for clinical use, in order to facilitate a broad range of applications (MORGAN et al., 1993; SUMMER-SMITH, 2002).

The bone banks employ various preservation methods. In the biological method, the grafts are maintained in the harvest bed in order to ensure revascularization prior to its implantation in the recipient bed. The physical method, on the other hand, uses low preservation temperatures. Initially, temperatures between 2°C and 5°C were used. However, the conservation time was limited to 3 weeks. In current practice, the grafts are frozen at –30°C, which allows for an unlimited period of use. Cryopreservation at temperatures below –120°C is also feasible for osteochondral grafts; however, cryoprotectant substances must be simultaneously applied. Patients implanted with allografts preserved by cryogenic techniques showed good tolerance. This, in association with the previously described advantages and shelf-life, justify the large-scale use of cryogenic preservation (CARVALHO et al., 1996).

Gioso et al. (2002) stated that bones immersed in 98% glycerin at room temperature for nine years would be automatically preserved, therefore alleviating the need for extra freezing protocols. They discovered that the growth of microorganisms in preserved samples was not statistically significant. They also reported that glycerin is an excellent means of bone tissue conservation, and could facilitate the implementation of a canine bone bank. Cavassani et al. (2001) stated that the preservation of bone fragment osteogenic activity by glycerin enables the establishment of bone banks for grafting.

Other commonly used methods include the demineralization method and the chemical method. In the latter, aqueous solutions of merthiolate (1:1000 and 1:5000) were applied or renewed every two weeks, respectively. Another method involves the conservation of grafts in alcohol, glycerin, and

beta-propiolactone solution. However, this method causes osteoinduction loss, and is hence rather disadvantageous (CARVALHO et al., 1996).

As with preservation, there are a number of techniques for the procurement and preparation of bone tissue grafts used by tissue banks, involving aseptic excision or excision in the absence of aseptic conditions. When aseptic conditions are not observed, secondary sterilization methods, such as irradiation, ethylene oxide or ethanol treatments, or autoclaving, are required (FEOFILOFF; JESUS-GARCIA, 1996; LAVERNIA et al., 2004). The grafts in the following conditions can be preserved: fresh material to be transplanted within 12 hours of harvest, frozen fresh material, lyophilized material that has been subjected to fat and bone marrow removal and subsequently vacuum-dehydrated and demineralized (MORENO; FORRIOL, 2002; MORGAN et al., 1993). The material must be harvested from the corpse as soon as possible post-mortem. On an average, material can be collected from a corpse refrigerated at –4°C up to 24 hours, or from a non-refrigerated corpse stored at 20°C up to 12 hours post-mortem (RONDINELLI et al., 1994; LAVERNIA et al., 2004). According to Stefani et al. (1989), tissues can be harvested in a sterile manner only when the appropriate surgical procedures are applied. These procedures, although labor intensive, would eliminate the need for secondary sterilization, which could interfere with the biological and mechanical properties of the tissues.

Diaphyseal cortical grafts are obtained from the long bones by sectioning the metaphyseal portion of each end, curettage, and medullary cavity washing for the removal of soft tissues (BRINKER et al., 2009; SUMMER-SMITH, 2002), or by harvesting, preserving, and osteotomizing of entire long bones at the time of use (FEOFILOFF; JESUS-GARCIA,1996).

Bone tissue banks typically preserve material in sterile conditions at temperatures below –40°C for periods longer than 6 months, or between –18°C and –12°C for shorter periods. No changes have been reported in the physical or mechanical properties of grafts stored at –20°C. On the other hand, samples cryopreserved at –80°C displayed a reduction in the cortical bone rigidity (MORENO; FORRIOL, 2002). In addition, Morgan et al. (1993) have reported that bones preserved fresh or those frozen at –20°C may be used for several months, with a possibility of slow degradation. On the other hand, samples preserved at –70°C do not demonstrate any degradation, and can be preserved for several years.

Feofiloff and Jesus-Garcia (1996) attributed several advantages to the use of preserved allografts as opposed to the use of autogenous grafts. These included reduced duration of surgery and anesthesia, blood loss, risk of complications such as infections, hematomas, and vascular and nerve injuries, local donor site instability, and cosmetic deformity, in addition to chronic pain attributed to the donor site. Weyts et al. (2003) reported that the osteoinductive properties of frozen bones could be primarily attributed to the dead bone matrix that might provide osteoblast-stimulating growth factors and other essential proteins, and/or an osteoclast substrate to direct bone remodeling. Recently, however, it has been suggested that some cells in bone biopsies may survive the standard bone bank freezing protocols.

One of the major complications associated with the bone grafting process is the risk of infection during the postoperative period, which could culminate in implant failure. The implants may be contaminated during harvest, storage, or at the time of implantation (FARRINGTON et al., 1998). In order to reduce the rate of contamination, a strict control must be followed throughout the process. In addition to testing for donor selection, a bone graft can be subjected to microbiological analysis (by collecting surface material at the time of harvest using a swab sterilized in Stewart's medium) in aerobic and anaerobic cultures. All fragments must be quarantined at a temperature below –20°C until the results are evaluated. In order to reduce the risk of disease transmission, the bone bank must be subjected to periodic safety checks. A general rule

to be followed is that 10% of the contents in the bank must be cultured every 6 months (MORGAN et al., 1993).

Liu et al. (2002) reported an infection rate of 7.8% in allografts. The allografts were determined to be contaminated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Sutherland et al. (1997) described the presence of *Klebsiella oxytoca* in addition to the two aforementioned bacteria, at a contamination rate of 12.2%. Approximately half (50%) of the implants using contaminated bone grafts resulted in failure. Boerlin et al. (2001) state that *Staphylococcus intermedius* is one of the four major pathogens found in veterinary hospitals, along with *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Enterococcus faecium*.

Eugster et al. (2004) reported that the incidence of infection post-surgery was associated with three factors: the duration of surgery, a large number of people moving within the operating room, and insufficient cleansing and disinfection of the operating room.

Roos et al. (2000) had insisted on the importance of the maintenance of local bone tissue banks, where orthopedic interventions could be performed. This is because of the numerous benefits of the procedures that require large quantities and different anatomical forms of preserved materials.

Oliveira et al. (2013) reported the implementation of a canine cortical bone bank, preserved in 98% glycerin, at the Veterinary Hospital of the Faculdade Integrado de Campo Mourão, which enabled the performance of reconstructive orthopedic surgery to treat bone loss in canine patients (OLIVEIRA et al., 2013).

The aim of this study was to evaluate the feasibility of maintaining a canine bone bank (*Canis familiaris*) by quantifying the bacterial contamination of bone grafts submitted to cryogenic preservation (-24°C), and through monthly assessment of the bacteriological cultures over a period 6 months.

Material and Methods

The humerus, radius, ulna, femur, tibia, and fibula of 10 animals, euthanized for reasons unrelated to this project, were collected. The biological material was harvested by a local trichotomy procedure, the skin antisepsis was ensured using a germicide solution of PVP and alcohol, and surgical fields were placed. The bones were removed with the aid of conventional surgical instruments, from longitudinal skin incisions in the fore and hind limbs. The muscle layers, ligaments, and tendons were cut and removed. The bones were dismantled and removed. Drapes, gloves, and scalpel blades were exchanged for the harvesting of bones in the contralateral limb.

The bones were subjected to a skeletonization process, according to the method described by Feofiloff and Jesus-Garcia (1996). This method consisted of removing all the soft parts and using a sterile surgical cloth for each graft, thus avoiding cross-contamination. After skeletonization, the samples from all bones were swabbed with Stewart's medium and processed for bacteriological cultures.

The bones were washed with a sterile saline solution and packed separately in two special sterile plastic bags for bone banks (Sarstedt AG & Co., Numbercht, Germany; No/ref: 86.1198, 300 × 500 mm), one inside the other, which were properly identified and sealed with nylon clamps. The grafts were frozen at –24°C in a vertical Bosch Intelligent 32 Freezer (Bosch, Stuttgart, Germany). The total operating time (from time of death to freezing) and the exposure time to the environment for each graft (from bone disarticulation to freezing) was recorded.

Over the next 6 months, 10% of the samples were chosen at random for monthly bacteriological evaluation of the grafts. Microbiological examination of the samples included culturing in BHI broth (Brain and Heart Infusion), sheep's blood agar (5%), and MacConkey agar, aerobic incubation at 37°C, with readings noted every 24-96 hours. The

isolated microorganisms were identified according to the method detailed by Lennette et al. (1985), and classified according to the guidelines set by Krieg and Holt (1994) and Murray et al. (1999).

Results

A hundred bones were collected, out of which 94 were submitted to the bone bank. The mean operative time was 4 hours and 22 minutes, the exposure time for each bone was approximately 19 minutes and 39 seconds, and the contamination rate was 10%.

The 94 preserved grafts were subjected to bacteriological culture immediately after harvest, with a 10.6% percentage of contamination. *Bacillus* sp., *Staphylococcus epidermidis*, *Staphylococcus intermedius*, and other gram-negative non-fermentative bacteria were observed. The bones obtained from animals 01 and 03 were contaminated. No correlation was found between contamination and the total surgical time or the time of exposure of each graft to the environment.

Monthly assessments of bacterial contamination were conducted on the preserved grafts. Ten percent of the grafts obtained from each animal were assessed, as the collections were carried out on different dates and the grafts preserved for different periods. In all, 46 grafts were assessed post-freezing and 100% of the cases were observed to be negative for bacterial contamination.

Discussion

The samples were cryogenically frozen at –24˚C based on the observations of Feofiloff and Jesus-Garcia (1996), who reported the advantages of using preserved allografts, and Weyts et al. (2003), who reported the maintenance of the osteoinductive properties of frozen bones. The advantages of using preserved allografts included reduced surgical time, blood loss, and potential complications of the donor donation.

The harvesting technique chosen for mounting of the bone bank was considered effective with respect to the harvest time and control of bacterial infection, convenience, and low cost, parameters considered to be ideal by Morgan et al. (1993) and Summer-Smith (2002).

The 94 preserved grafts were contaminated at a rate of 10.6%, which was considered acceptable when compared to the data published by Liu et al. (2002) and Sutherland et al. (1997), who obtained a contamination rate of 7.8% and 12.2%, respectively. Of the ten animals used, positive contamination results were obtained in animals 01 and 03 only, with 40% and 60% contamination, respectively. All the bones expressing positive bacteriological results were discarded. This was based on the regulations set by Liu et al. (2002) and Sutherland et al. (1997), who demonstrated that contaminated grafts result in a 50% failure after grafting despite being subjected to cryogenic preservation.

Two types of microorganisms were identified in animal 01: *Staphylococcus epidermidis*, and a gram-negative non-fermenter. *Staphylococcus epidermidis* colonizes mainly in the skin, and the contamination could be explained by insufficient antisepsis of the pre-operative donor skin. Bacteria belonging to the gram-negative non-fermenting class were mainly found in the environment.

Two types of contaminating microorganisms were identified in animal 03: *Bacillus* sp. and *Staphylococcus intermedius*. *Staphylococcus intermedius* is one of the main colonizers of the epidermis, which would therefore be the most likely contamination route for this graft. Boerlin et al. (2001) identified *Staphylococcus intermedius* as one of the four major pathogens found in veterinary hospitals, along with *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Enterococcus faecium*. The *Bacillus* sp. is predominantly found in the environment. We believe that this high level of contamination during the harvest could have been due to the use of air conditioning, as this was the only harvest conducted in an air conditioned room. In

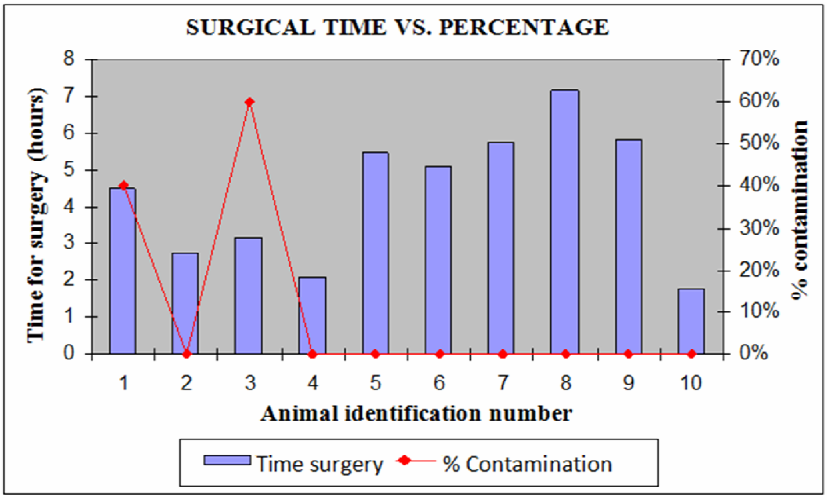
addition, data provided by the engineering company Cabano indicated a major cause of contamination in surgical procedures to be environmental bacteria, with the members of the surgical team themselves being major carriers. Furthermore, incorrect maintenance of the air conditioning system can also lead to an increase in environmental bacteria, because of bacterial accumulation on the filters, which migrate back to the environment with the air that is circulated in the operating room (RODRIGUES JUNIOR, 2003).

With the identification of 40% contamination in animal 01, some changes were effected, in order to eliminate the identified points as possible sources of contamination, in accordance with the recommendations of Stefani et al. (1989) (use of surgical sterilization techniques and a surgical environment for the harvest of grafts). The harvest time of each bone was monitored in order to correlate the contamination with prolonged manipulation of the graft. Similarly, Eugster et al. (2004) had concluded that the incidence of infection in the postoperative period was associated with three factors: the duration of the surgery, a large number of people moving within the operating room, and improper cleansing and sterilizing of the operating room.

The surgical team consisted of two members for the harvest of graft, one for skeletonization, and one non-sterile member as a general assistant. The number of people in the team allowed for a reduction in the surgical time, reduced graft exposure to the environment, reduced risk of contamination, and improved harvest efficiency, according to Eugster et al. (2004) and Farrington et al. (1998).

We concluded that there was no relationship between the time of exposure to the environment and the presence/absence of contamination. Animal 03 presented a 60% contamination rate, but had a lower exposure time to the environment compared to the uncontaminated animals 02 and 04 (who had the highest average exposure time). Based on this result, we concluded that the time of environment exposure used in this study can be considered ideal. Therefore, we state that an average exposure time of 20 minutes could be sufficient to avoid environmental contamination in a surgical environment. The same correlation characteristics were observed between contamination and total surgical time (time elapsed from death to the end of the harvest), as shown in Figure 1. In this case, animal 03 displayed the fourth lowest total surgical time, while the animals operated on for longer periods presented no contamination.

Figure 1. Relationship between the total time for surgery and presence of contamination among the 10 animals evaluated in the study.



Animals 05 to 09 showed the highest total surgical harvest time. However, the average total surgical time of 4 hours and 22 minutes was maintained. Lavernia et al. (2004) and Rondinelli et al. (1994) mandated that the harvest time should be, on average, within 24 hours post-mortem, when the corpse is stored at –4°C, or within 12 hours post-mortem without cooling, (a temperature of 20°C). As seen in Chart 1, even the animal with the highest average surgical time (7 hours and 11 minutes) was within this time range, in agreement with literature.

Carvalho et al. (1996) reported that the grafts preserved at –30°C presented an unlimited period for use. In turn, Moreno and Forriol (2002) concluded that grafts preserved at temperatures below –40°C under sterile conditions could be used for longer than 6 months, or for shorter periods when preserved between –18°C and –12°C. The bone bank created in this study had a –24°C storage temperature. Therefore, we validated the viability of grafts for a period of 6 months.

The technique used in this study enables the deployment of a bone bank in places that do not have many available resources, as it is a low-cost investment (utilizing a common freezer reaching a temperature below –24°C), compared to the cost of a freezer that reaches a temperature below –80°C.

Conclusion

The techniques chosen for the deployment of the bone bank were effective and feasible. The viability of the bones over a period of 6 months and a 100% sterility rate of the grafts was confirmed.

Ethics and biosafety committee 3048/2013.

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