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BASIC RESEARCH

COMPARATIVE STUDY OF CRYOPRESERVED BONE TISSUE AND TISSUE PRESERVED IN A 98% GLYCEROL SOLUTION

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OBJECTIVE: To compare the bone graft cryopreservation method (at -80ºC) with a preservation method using a 98% glycerol solution at room temperature (10ºC-35ºC), by testing the antibacterial and fungal effects of 98% glycerol and comparatively analyzing the observed histological changes resulting from the use of both methods.

METHOD: This study was of 30 samples of trabecular bone tissue from 10 patients undergoing total hip arthroplasty. Each femoral head provided 3 samples that were randomized into 3 groups, namely, the control group, the cryopreserved group, and the group preserved in a 98% glycerol at room temperature for 1 year. The samples were submitted to histomorphologic, cell feasibility, and microbiologic analyses. The results were statistically analyzed using the McNemar test, with a statistical significance index of 0.05.

RESULTS: Values obtained using the McNemar test to compare probability distributions of histomorphologic variables (mature or lamellar bone, immature bone, and necrosis) and cell feasibility (osteoblasts and osteoclasts) indicated that there is no difference between the distributions of variables under the 3 experimental conditions. Microbiological analysis of the 98% glycerol solution and bone fragments from samples stored for 1 year at room temperature did not show bacterial or fungal growth. The histological and microbiological investigation were performed at 2 different time points: immediately after the sample processing and after 1 year.

CONCLUSION: The method used to preserve bone grafts kept in 98% glycerol at room temperature (10ºC-35ºC) was similar to cryopreservation in terms of bone matrix preservation; no bacteria or fungi were found in the samples.

KEYWORDS: Bone tissue. Glycerol. Bone transplant. Cryopreservation. Bone graft.

INTRODUCTION

The need to use allografts intended to surgically fill bone losses is quite old. Autologous grafts are often used to repair injured structures, although it is not always possible to obtain them in the desired quantity and quality. For this reason, homologous grafts appeared as an alternative to enrich the therapeutic arsenal, replacing the autologous grafts in the treatment of many orthopedic affections requiring replacement of large amounts of bone tissue.[1-5]

Allograft storage is but one stage to be controlled within the entire process that culminates with the provision of the graft for transplant. For each stage, technical and quality criteria have been established within the methodology currently required for the musculoskeletal system tissue bank to work, and such criteria were strictly met.

Freezing is currently the method of choice for allograft preservation, since it reduces the immunogenic potential while preserving the biomechanical and osteoinductive properties. In addition to inhibiting bacterial growth, fro-

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zen speciments maintain bone matrix integrity and remain in good condition to be transplanted later. However, it is a quite complex and expensive method.[3,6-10]

The purpose of performing a study involving an alternative method of bone graft preservation was to make available a method as effective as cryopreservation, although much less expensive, since this would allow for storage of the allografts at room temperature and facilitate their transportation to the surgical site.

Various reports describe the use of highly concentrated glycerol solutions as an alternative medium for preserving homologous tissues stored at room temperature. It has been claimed that such methodology does not alter the fundamental features of tissues, maintaining the architecture of the graft for at least 1 year. Moreover, glycerol has bactericidal and fungicidal properties and inhibits the onset of an immune response to foreign matter. However, the literature concerning orthopedics does not refer to preservation of osteofascial-chondral-ligamentous tissues with this agent. [5,11-16].

The objective of this study was to test a 98% glycerol solution for antibacterial and antifungal effects when bone tissues are kept at room temperature (10ºC -35ºC). A comparative analisys of histological changes resulting from cryopreservation versus 98% glycerol solution was also underrtaken.

METHOD

This study used 30 samples of trabecular bone tissue from 10 patients undergoing total hip arthroplasty (THA) performed by the Hip Group of the Institute of Orthopedics and Traumatology, Hospital das Clínicas, Faculty of Medicine of the University of Sao Paulo, Brazil. The femoral heads were included in this research project after the patients gave their explicit informed consent. The samples were identified by numbers in ascending order according to surgery dates.

Samples were collected in a surgery room of the Institute's Tissue Bank provided with vertical laminar flow and Class 100 absolute HEPA (high efficiency particulate air) filters. Rigorous aseptic technique was used throughout every procedure.

Of the 30 samples, 10 formed the control group; 10 were subjected to cryopreservation (at -80ºC), while another 10 were preserved in 98% glycerol solution for 1 year.

The histological and microbiological investigation were performed at 2 different time points: immediately after the sample processing and after 1 year. Aerobic and anaerobic bacterial cultures, as well as fungal cultures, were performed. Because the method has already been established and used in many centers, we chose not to microbiologically analyze the cryopreserved samples.

All samples were submitted to histomorphologic and cell feasibility analysis. Microscopy was used to analyze the amount of osteoblasts, osteoclasts, osteocytes, and fibroblasts, as well as the presence of immature and lamellar bone, nonmineralized osteoid, and necrosis. This analysis allowed us to evaluate cell feasibility and bone matrix maintenance. For tissue analysis, we established the following scores: absent (#), reduced (+), normal (++), and increased (+++) (Table 1); for the cell feasibility analysis, the scores were absent (#) and normal (+) (Table 2).

The statistical analysis considered that the experimental units were 10 subjects, each one of them providing 3 bone slices (observation units) that were individually subjected to a different experimental condition. So, the study followed a plan in which bone slices of the same individual were observed under 3 different experimental conditions, generating 3 samples that may be not independent. For each given bone slice from and individual that was observed in the control group,

Table 1 - Histomorphologic description of samples

HISTOMORPHOLOGY												
SAMPLE #	CONTROL (Gc)				CRYOPRESERVED (Gcryo12)				GLYCEROL 98% (Gg12)			
	MB	IB	OST	NEC	MB	IB	OST	NEC	MB	IB	OST	NEC
1	++	#	#	+++	++	#	#	+++	++	#	#	+++
2	++	+	#	#	++	#	#	+++	++	#	#	+++
3	++	#	#	#	++	#	#	+++	++	#	#	+++
4	++	#	#	#	++	+++	#	+++	++	+++	#	+++
5	++	+++	#	+++	++	+++	#	+++	++	+++	#	+++
6	+++	+++	#	#	+++	+++	#	#	+++	+++	#	#
7	+++	#	#	#	+++	#	#	#	+++	#	#	#
8	++	+++	#	+++	++	+++	#	+++	++	+++	#	+++
9	++	#	#	#	++	#	#	+++	++	#	#	+++
10	++	+++	#	+++	+++	+++	#	+++	++	++	#	+++

SCORE: # : absent; + : reduced; ++ : normal; +++ : increased; MB: mature or lamellar bone; IB: immature bone; OST: nonmineralized osteoid; NEC: necrosis

Table 2 - Cell feasibility description of samples

SAMPLE #	CONTROL (Gc)				CRYOPRESERVED (Gcryo12				98% GLYCEROL SOLUTION (Gg12)			
	OB	OC	OST	FB	OB	OC	OST	FB	OB	OC	OST	FB
1	+	+	+	+	#	#	+	+	#	#	+	+
2	+	+	+	+	+	#	+	+	+	#	+	+
3	+	+	+	+	+	#	+	+	+	#	+	+
4	+	+	+	+	+	#	+	+	+	#	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	#	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	#	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+

SCORE: # : absent; + : normal; OB: osteoblasts; OC: osteoclasts; OST: osteocytes; FB: fibroblast; Gc: control group; Gg12: 98% glycerol solution group; Gcryo12: cryopreserved group

corresponding slices will exist in the cryopreserved and glycerol groups, so that we had paired samples.

Initially, we developed tables including frequency distributions and percentages of the morphology and cell feasibility variables under each of the experimental conditions (control, cryopreservation, and glycerol). These conditions were compared in terms of probability of occurrence of the category of each variable. These comparisons were made using paired conditions (control versus cryopreservation, control versus glycerol, and cryopreservation versus glycerol), using the McNemar test (Fisher and van Belle, 1993). This technique takes into account the pairing adopted to conduct the study. Since the sample size was small, the precise distribution of the test statistics was considered. The global significance level of the comparison of each variable under each of the 3 experimental conditions was established as 0.05.

RESULTS

The microbiologic analysis of the 98% glycerol solution and bone fragments from samples stored for 1 year at room temperature did not show bacterial or fungal growth.

Histomorphologic description of samples and cell feasibility data are presented in Tables 1 and 2. We did not test the nonmineralized osteoid variable because the values assumed by this variable did not vary (the category was observed in all slices). The descriptive levels indicated that there were no differences between the distributions of the following variables: mature bone, immature bone, and necrosis under the 3 experimental conditions.

We did not perform hypothesis tests for the cell feasibility variables, osteocytes and fibroblasts, since their results did not vary, with all slices falling in a normal category. Also, we did not compare distribution of osteoblasts in the controls versus the cryopreserved and glycerol groups because the control group presented normal results in all samples. The results of the statistical analysis indicated that there was no difference between the distributions of the variables, osteoblasts and osteoclasts, under the two storage conditions. The frequency distributions, percentages of the variables, and results of hypothesis testing under the 3 experimental conditions are shown in Tables 3, 4, and 5.

The absolute and relative frequencies of the scores in mature or lamellar bone are presented in Table 3.

Table 3 - Absolute and relative frequencies of the scores in mature or lamellar bone

	Group Control		Cryopreserved		98% glycerol	
Score	n	%	n	%	n	%
Normal	8	80.0	7	70.0	8	80.0
Increased	2	20.0	3	30.0	2	20.0

The *P* values obtained by the MacNemar test for histomorphometric variables are shown in Table 4. Concerning the immature bone (IB) variable, the reduced, normal, and increased categories were grouped into a single category so that the test could be applied. The probability levels (*P*) indicate that there were no differences between the distributions of the mature bone (MB), immature bone (IB) and necrosis (NEC) variables under the 3 experimental conditions.

As previously mentioned, the cell feasibility analysis did not include hypothesis testing for osteocytes (OSTs) and fibroblasts (FBs) because for these two variables the normal category was observed in all slices under the 3 experimental conditions. The probability levels achieved in the analysis of these two variables are shown in Table 5. Our conclusion was that the control, cryopreservation, and glycerol groups did not differ in terms of distribution of osteoblasts (OBs) and osteoclasts (OCs); the probabilities

Table 4 - *P* values for the McNemar test in the comparison of probability distributions of histomorphologic variables under 3 experimental conditions

Variable	Control versus Cryopreserved	Control versus Glycerol	Cryopreserved versus Glycerol
MB	> 0.999	> 0.999	> 0.999
IB	> 0.999	> 0.999	> 0.999
NEC	0.375	0.375	> 0.999

MB = mature or lamellar bone; IB = immature bone; NEC = necrosis

Table 5 - *P* values for the McNemar test in the comparison of probability distributions of cell feasibility variables under 3 experimental conditions

Variable	Control versus Cryopreserved	Control versus Glycerol	Cryopreserved versus Glycerol
OB	> 0.999	> 0.999	> 0.999
OC	0.188	0.096	0.500

OB = osteoblasts; OC = osteoclasts

of occurrence of the absent or normal categories of OBs and OCs are equal under the 3 experimental conditions.

DISCUSSION

In orthopedics and dentistry, interest in development of techniques to compensate for bone loss increases every day. Therefore, allografts have been chosen as the main source of bone tissue supply and have become an alternative to the use of autologous grafts.

The first step in obtaining allografts consists of a rigorous selection of the donor. After the screening of donors, the next step is tissue removal using suitable surgical technique and processing, followed by the final storage stage.

Currently, the most frequently used preservation method for bone tissues is cryopreservation, that is, the use of low temperatures (minimum –80º C). The use of this method involves high costs because, apart from the intrinsic value of the freezing units, indirect costs such as preventive maintenance, maintenance of a properly refrigerated room, 24-hour/day alarm systems, and power generators in case of lack of power must also be considered.

Glycerol at high concentrations is known to have antibacterial, antifungal, and antiviral action. Several studies have shown that a 98% solution of glycerol at room temperature is effective in preserving biological tissues.[11,18-23]

However, there are no studies in the orthopedic literature regarding preservation and storage of osteofascial-chondral-ligamentous tissues with this agent. Only Pigossi (1964) and Backere (1994) have suggested the use of this method to preserve bones.

In order to ensure that the process of allograft bone integration will effectively occur, the status of bone matrix preservation is fundamental. In both preservation methods examined in this study, this matrix was kept preserved, and no statistically significant changes were found. This important aspect is related to the preservation method, since the bone matrix (not the cell feasibility) is a desirable item in bone allograft.[24]

Another essential aspect of bone preservation for allografts is the absence of growth of bacteria and fungi in the preserved samples. The results with glycerol preservation/room-temperature storage are similar to those obtained with the cryopreservation method at – 80°C. However, further studies must be performed to evaluate other items such as:

- Maintenance of the bone matrix osteoinductive capacity;
- Cell-mediated immune response to the allograft, because the preserved cells may retain recognizable alloantigens;
- Biomechanical properties, which play an important role in the therapeutic success of transplants. It is fundamental that the preservation method used does not compromise the rigidity of allografts and the forces acting on them.
- Removal of glycerol from the bone tissue and measurement of the quantity retained in the graft, in order to determine the levels of tolerance of this tri-alcohol.

CONCLUSIONS

1) There was no bacterial or fungal growth in samples stored for 1 year in a 98% glycerin solution at room temperature (10 ºC -35ºC).
2) Both preservation methods were similar in terms of bone matrix preservation.

RESUMO

Giovani AMM, Croci AT, Oliveira CRGCM, Filippi RZ, Santos LAU, Maragni GG. Estudo comparativo entre o tecido ósseo criopreservado e o conservado em glicerol a 98%. Clinics. 2006;61(6):565-70.

OBJETIVO: Comparar o método da criopreservação de enxertos ósseos (– 80° C) com o da conservação em glicerol a 98% em temperatura ambiente (10º C a 35º C), testando os efeitos antibacterianos e antifúngicos do glicerol a 98% e analisando comparativamente as alterações histológicas verificadas e decorrentes do emprego dos dois métodos.

MÉTODO: Este estudo foi constituído de 30 amostras de tecido ósseo trabecular provenientes de 10 pacientes, submetidos a Artroplastia Total do Quadril. Cada cabeça femoral forneceu 3 amostras e estas foram divididas aleatoriamente em 3 grupos, a saber: controle, criopreservado e conservado em glicerol a 98% à temperatura ambiente durante um ano. As amostras foram encaminhadas à Anatomia Patológica para estudo histomorfologico, de viabilidade celular, e microbiológico. Os resultados foram analisados estatisticamente pelo método de McNemar, com índice de significância de 0,05.

RESULTADOS: A análise dos valores obtidos no teste de McNemar na comparação das distribuições de probabilidades das variáveis da histomorfologia (osso maduro ou lamelar, osso imaturo e necrose) e da viabilidade celular (osteoblastos e osteoclastos) indica não haver diferença entre as distribuições das variáveis nas três condições experimentais. A análise microbiológica da solução de glicerol a 98% e dos fragmentos ósseos das amostras armazenadas durante um ano em temperatura ambiente não apresentou crescimento bacteriano ou de fungos. As espécimens do grupo controle foram analisadas histológica e microbiologicamente logo após a coleta das mesmas.

CONCLUSÃO: O método de conservação de enxertos ósseos mantidos no glicerol a 98% em temperatura ambiente (10ºC a 35ºC) foi similar ao da criopreservação quanto à preservação da matriz óssea e à ausência de crescimento de bactérias ou fungos.

UNITERMOS: Tecido ósseo. Glicerol. Transplante ósseo. Criopreservação. Enxerto ósseo.

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