

Comparative study and histomorphometric analysis of bone allografts lyophilized and sterilized by autoclaving, gamma irradiation and ethylene oxide in rats¹

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ABSTRACT

PURPOSE: To compare three sterilization methods (autoclave, gamma irradiation and ethylene oxide) over non demineralized lyophilized bone allografts.

METHODS: Bone allografts were implanted on paravertebral muscles of 21 rats. After 30 days animals were sacrificed and grafts underwent comparative analysis regarding histomorphometric and macroscopic parameters.

RESULTS: Allografts that underwent the three sterilization methods presents similar weight gain, cortical thickness similar to control group, and less fibrosis than the control group. Grafts that underwent sterilization in autoclave presented less presence of multinucleated giant cells, although not statistically significant. There was also no statistically significant difference regarding mineralization on the three groups.

CONCLUSION: The three sterilization methods cause similar effects on bone allografts regarding macroscopic and histomorphometric parameters.

Key words: Bone and Bones. Transplantation. Homologous. Osteogenesis. Sterilization. Rats.

Introduction

The difficulties in surgical repair of craniofacial deformities require that new techniques be developed to achieve better functional and esthetic results. Notwithstanding the surgical techniques developed in the sixties¹, new procedures for bone grafting are necessary, because of their importance in the restoration of the craniofacial bone structure.

The enhanced knowledge of physiological factors related to the integration of bone grafts has allowed the use of various types of grafts with increasingly consistent results.

The use of bone allografts (from another individual of the same species), not only saves surgery time because of the availability in bone banks, but also decreases the procedure's morbidity. This type of bone graft promotes osteogenesis due to its osteoinductive potential requiring preparation to reduce the antigenic load and the risk for transmission of infectious diseases²⁻⁵.

To date there is no consensus regarding the ideal sterilization to ensure preservation of the allograft potential of osteoinduction and ability for integration⁵⁻¹⁰. The main methods of sterilization of biological materials used today are autoclave, gamma irradiation and ethylene oxide. The most suitable method would seem to be the one that causes less damage to the osteoinductive property.

Thus, the purpose of this experimental work is to compare the effect of these three sterilization methods on lyophilized bone grafts using macroscopic parameters and histomorphometric measurements for the assessment of osteogenesis.

Methods

In the study 42 male, isogenic, adult Wistar rats were used. Initially, 21 rats were sacrificed and within three hours the 42 tibiae were extracted from these animals, and the soft tissue and periosteum were removed. Then bones were severed crosswise in the middle section so as to obtain the proximal half of about 25mm, frozen and then lyophilized for 24 hours and weighed on a precision balance before and after this procedure.

The segments were then divided into two groups. The first, called Group 1 (control) was subdivided into three subgroups:

- Subgroup 1a (control of subgroup 2a);
- Subgroup 1i (control of subgroup 2i);
- Subgroup 1o (control of subgroup 2o).

The second group, called Group 2 was subdivided into three subgroups according to the sterilization method

- Subgroup 2a - sterilization method was autoclave

(128°C for 40 minutes);

- Subgroup 2i - sterilization method was gamma irradiation (Cobalt -60 to 2.5 mRads);

- Subgroup 2o - sterilization method was ethylene oxide (for 6 hours).

After undergoing the three different methods of sterilization the bone segments were hydrated in saline solution and then implanted in the left paravertebral muscles at level of the lumbar region of the 21 recipient rats, and on the right side were implanted the segments of the corresponding group 1 (control).

All animals received intraperitoneal tetracycline (Oxytetracycline Pfizer) 20mg/kg on the 2nd and 28th days of the experiment. The grafts were removed after 30 days, and again weighed on a precision balance and sent for histological analysis. All slides were treated alike and were analyzed under a magnification of 250 times with the same microscope, by the same observer, without identification of the origin. The parameters studied were trabecular volume, fibrosis, cortical thickness, presence of multinucleated giant cells and mineralization.

Measurements were performed on 15 fields in the area of the proximal epiphysis of the tibiae, immediately distal to the epiphyseal growth plate and equidistant from the two cortices.

All data were statistically analyzed. For the variable weight the Kruskal-Wallis test was used. As for the quantitative variables (trabecular volume, cortical thickness and fibrosis) and the semi quantitative (presence of multinucleated giant cells) minimum and maximum values were identified, the median, mean and standard deviation were calculated for each of the subgroups under study. In the comparative analysis of the three types of sterilization utilized, with respect to these variables, the following formula was used to calculate the percentage variation (PV):

$$PV = \frac{[\text{sterilized} - \text{control}]}{\text{Control}} \times 100$$

The values obtained were submitted to the same statistical test. For analysis of the variable mineralization, contingency tables were employed and the Fischer's t test was used to compare the three types of sterilization.

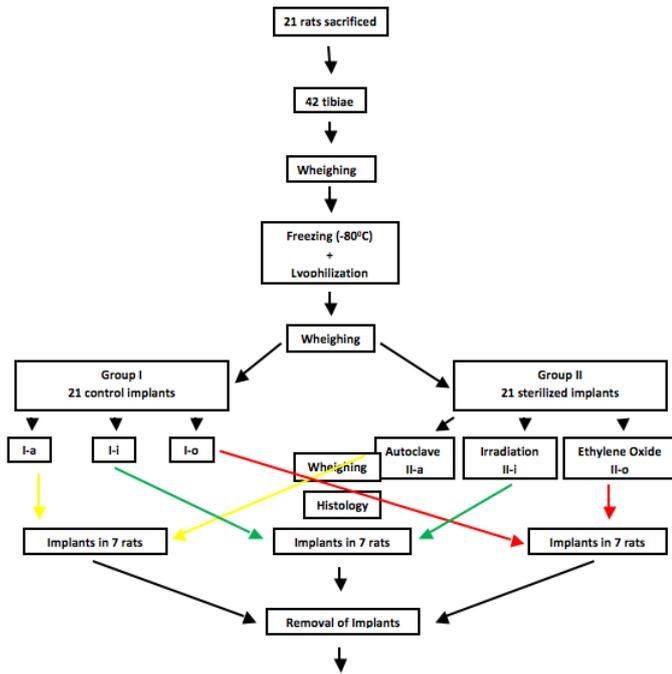


FIGURE 1 - Stages of the experiment.

Results

Macroscopic (weight) and histomorphometric analysis of the segments studied showed the following results:

Comparing the three sterilization methods, regarding the weight of bone allografts, it was found that all segments showed an increase in weight after grafting, regardless of the sterilization method used (Table 1). This increase was found to be similar, with no statistically significant difference between subgroups.

TABLE 1 - Descriptive measurements of the variable weight (grams) and result of the comparison between the three sterilization methods.

Subgroup	Minimum	Maximum	Median	Mean*	Standard Deviation	P*
pre-graft weight (g)						
2-a	0.15	0.28	0.21	0.21	0.05	
2-i	0.15	0.24	0.22	0.21	0.035	
2-o	0.19	0.38	0.26	0.27	0.06	
post-graft weight (g)						
2-a	0.18	0.39	0.25	0.27	0.07	
2-i	0.19	0.33	0.28	0.27	0.05	
2-o	0.28	0.48	0.34	0.36	0.07	
Weight variation (%)						
2-a	14.29	47.06	33.33	11.14	-	
2-i	20.83	37.50	27.27	9.93	-	0.8313
2-o	11.54	47.83	33.33	11.93	-	

*Descriptive probability level for the Kruskal-Wallis test
For weight variation, the mean presented refers to the scores.

Analysis of the variable trabecular volume, disclosed that sterilized allografts showed higher median values than their controls. The sterilized grafts submitted to gamma irradiation had

a lower percentage change in this index than those submitted to the autoclave and ethylene oxide. This difference also was not statistically significant (Table 2).

TABLE 2 - Descriptive measurements of the variable trabecular volume (%) and result of the comparison between the three sterilization methods.

Subgroup	Minimum	Maximum	Median	Mean*	Standard Deviation	p*
Trabecular volume (%) – control						
1-a	11.63	18.60	14.00	14.74	2.87	
1-i	12.79	18.10	15.53	15.51	1.88	
1-o	10.90	23.97	17.90	17.45	4.87	
Trabecular volume (%) – sterilized						
2-a	10.40	25.03	19.80	18.53	5.21	
2-i	15.06	24.20	19.40	19.62	3.61	
2-o	12.68	27.90	21.63	21.27	5.21	
Variation of trabecular volume (%)						
METHOD						
autoclave	-15.65	77.78	17.20	11.00	-	
irradiation	6.81	89.21	11.98	11.00	-	1.0000
oxide	-9.68	87.37	18.20	11.00	-	

*Descriptive level of probability for the Kruskal-Wallis test
For variation of trabecular volume, the mean presented refers to the scores

In the analysis of variable fibrosis, the sterilized allografts showed lesser quantity of fibrosis than their controls, explaining the negative values of percentage variation. Comparing the three sterilization methods, it was found that the largest percentage variation in the median occurred in allografts submitted to the autoclave, but differences were not statistically significant (Table 3).

TABLE 3 - Descriptive measurements of the variable fibrosis (%) and result of the comparison between the three sterilization methods.

Subgroup	Minimum	Maximum	Median	Mean*	Standard Deviation	p*
fibrosis (%) – control						
1-a	0.00	74.90	28.10	27.69	26.41	
1-i	0.00	45.53	36.73	28.17	18.25	
1-o	6.80	67.13	30.77	31.25	22.37	
fibrosis (%) – sterilized						
2-a	0.00	20.56	2.10	4.40	7.23	
2-i	0.00	46.76	24.96	21.90	19.42	
2-o	4.87	34.96	7.50	11.03	10.85	
variation of fibrosis (%)						
METHOD						
autoclave	-99.78	-	-84.21	8.71	-	
irradiation	-99.78	-	-8.10	13.57	-	0.3384
oxide	-90.45	184.23	-75.63	10.71	-	

*Descriptive level of probability for the Kruskal-Wallis test
For variation of fibrosis, the mean presented refers to the scores

With regard to cortical thickness, this showed practically no change when the three sterilization methods were compared with the control group, thereby explaining the median percentage variation values around zero (Table 4).

TABLE 4 - Descriptive measurements of the variable cortical thickness (microns) and result of the comparison between the three sterilization methods.

Subgroup	Minimum	Maximum	Median	Mean*	Standard Deviation	p*
Cortical thickness (μ) – control						
1-a	180.88	228.48	208.25	209.08	17.72	
1-i	154.70	195.63	172.55	175.51	15.85	
1-o	166.60	232.05	188.49	196.33	24.06	
Cortical thickness (μ) – sterilized						
2-a	210.10	230.86	214.20	216.94	7.34	
2-i	148.70	230.86	172.78	177.46	26.29	
2-o	163.03	229.62	199.92	199.23	21.90	
variation of cortical thickness (%)						
METHOD						
autoclave	-7.29	17.76	2.86	12.00	-	
irradiation	-10.76	22.78	-5.11	9.86	-	0.8094
oxide	-13.85	15.76	0.46	11.14	-	

*Descriptive level of probability for the Kruskal-Wallis test
For variation of cortical thickness, the mean presented refers to the scores

Regarding the presence of multinucleated giant cells, allografts of autoclave presented the lowest values among sterilized allografts, but comparing the percentage change with the controls there was no statistically significant difference (Table 5).

TABLE 5 - Descriptive measurements of the variable presence of multinucleated giant cells (0 to 4) and result of the comparison between the three sterilization methods.

Subgroup	Minimum	Maximum	Median	Mean*	Standard Deviation	p*
Multinucleated giant cells (0 a 4) – control						
1-a	0.0	4	3	2.14	1.77	
1-i	0.0	4	3	2.86	1.46	
1-o	2.0	4	4	3.43	0.79	
Multinucleated giant cells						
2-a	0.0	3	1	1.00	1.00	
2-i	1.0	4	3	2.71	1.25	
2-o	1.0	4	3	2.71	1.11	
Variation of multinucleated giant cells (%)						
METHOD						
autoclave	-75.0	-	-25.0	8.57	-	
irradiation	-75.0	-	0	14.00	-	0.2415
oxide	-50.0	33.33	-25.0	10.43	-	

*Descriptive level of probability for the Kruskal-Wallis test
For variation of multinucleated giant cells, the mean presented refers to the scores

Regarding the variable mineralization, the presence or absence of tetracycline marking was studied, mineralization was found to be more damaged in the subgroup of allografts from the autoclave (Table 6).

TABLE 6 - Presence or absence of mineralization in the subgroups studied (sterilized and controls).

Method	Lines of Mineralization				
	aa	ap	pa	pp	
autoclave	n	2	0	4	1
	%	28.57	0.00	57.14	14.29
irradiation	n	0	2	1	4
	%	0.00	28.57	14.29	57.14
Oxide	n	3	1	2	1
	%	428.6	14.29	28.57	14.29

aa = absent in sterilized as well as in control
ap = absent in control and present in the sterilized
pa = present in the control and absent in sterilized
pp = present in the control and present in the sterilized

The test comparing the three groups regarding the proportion of results that concerning damage to mineralization did not identify a statistically significant difference between the groups (Table 7).

TABLE 7 - Distribution of allografts according to the occurrence or not of damage to mineralization.

Method	Mineralization		
	with damage (aa, ap e pp)	without damage (pa)	
autoclave	n	4	3
	%	57.14	42.86
irradiation	n	1	6
	%	14.29	85.71
Oxide	n	2	5
	%	28.57	71.43

aa = absent in sterilized as well as in control
ap = absent in control and present in the sterilized
pa = present in the control and absent in sterilized
pp = present in the control and present in the sterilized

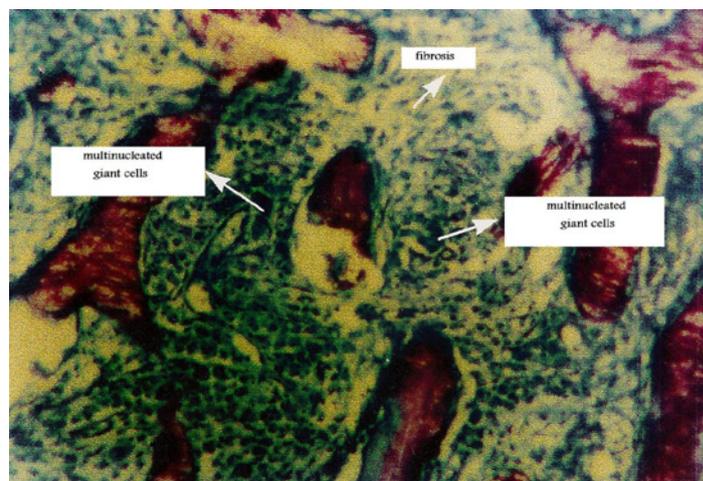


FIGURE 2 - Histological aspect of lyophilized allograft sterilized by autoclaving (250x). Subgroup 2-a.

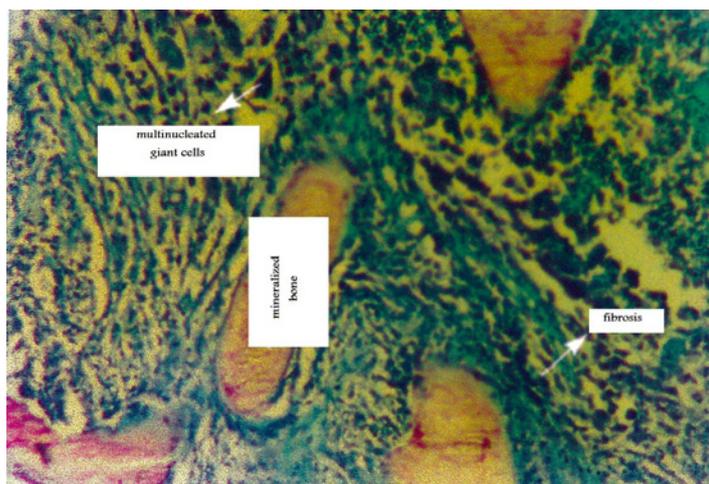


FIGURE 3 - Histological aspect of lyophilized allograft sterilized by gamma irradiation (125x). Subgroup 2-i.

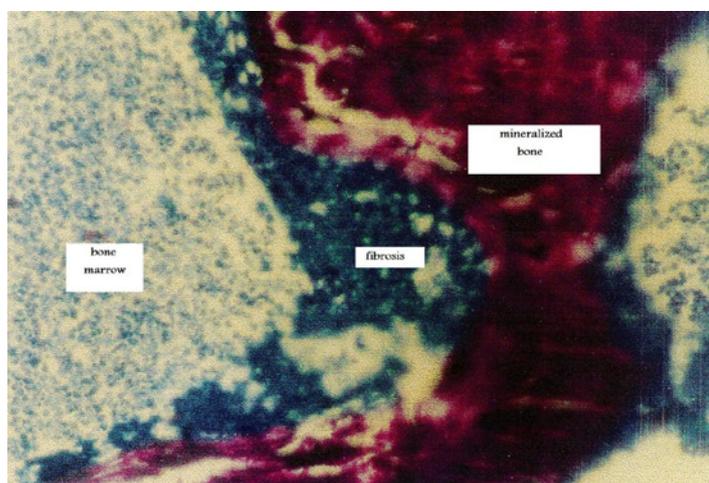


FIGURE 4 - Histological aspect of lyophilized allograft sterilized by ethilen oxide (125x). Subgroup 2-o.

Discussion

Bone is a specialized connective tissue which, besides being the support structure for all other tissues, provides protection for vital organs and bone marrow. It consists of three types of cells (osteoblasts, osteocytes and osteoclasts) and an intercellular material called bone matrix.

The bone matrix is comprised of an inorganic and an organic portion produced by osteoblasts, prior to their developing into osteocytes, the mature cells surrounded by mineralized tissue. Osteoclasts are responsible for bone resorption. Bone is a live and dynamic structure, which is constantly being remodeled.

At this time when organ and tissue transplants have become routine procedures, the bone allografts also began to be employed on a large scale, and currently after blood and skin, the bone is the live human tissue most often transplanted¹¹.

This encouraged the development of several studies in an effort to understand what takes place with this structure when it is transplanted and what is the best type of bone graft to be used^{8,9}.

There is consensus regarding two methods for conservation of bone allografts¹². Lyophilization, and freezing at -80°C¹², that both bring about destruction of the bone live cells and reduce their antigenic load.

The three sterilization methods compared here are those most widely used for non-biological and biological materials. Although, all have proven effective for this purpose⁵⁻⁸, however they do cause damage to biological materials, and due to the complexity and variety of factors there is no consensus on which is the best method.

Variations in measurements of weight and volume of the grafts as well as histological studies were possible only through experimental work. Using mathematical equations, the histomorphometric study allows the calculations of volume and surface, by means of information obtained from plane specimens of bone tissue. This enables quantitative evaluation of bone structures and provides data on the mechanisms involved in bone formation and resorption. This method further allows visualizing mineralization zones highlighted by tetracycline deposits providing information about the time related to bone formation and mineralization. For macroscopic examination of the grafts, weight was the only parameter studied.

At the initial stage of integration all bone grafts presented resorption². Allografts have a longer initial inflammatory response, causing delay in vascularization, with a consequent period of increased resorption and retarded onset of osteogenesis. The weight increase found in these groups is probably due to rehydration in the lyophilized bone segments as well as to invasion by blood vessels and significant presence of an inflammatory process.

If the variable trabecular volume provides the volume occupied by the bone locks, higher values obtained therefore mean lesser resorption. Although allografts submitted to gamma irradiation have a lower percentage variation in relation to the median when compared to allografts submitted to the other two sterilization methods, this difference was not significant.

As for fibrosis, the lower indices found in sterilized allografts in relation to their controls indicates that less cellular activity was present, which also leads to lower bone resorption. Allografts that underwent irradiation displayed higher levels of fibrosis, that even if not significant when compared with other methods explain the lesser weight gain and lower trabecular volume of this subgroup.

Glowacki¹³ demonstrated that multinucleated giant cells

have characteristics similar to those of osteoclasts. Sterilization by autoclave and ethylene oxide induced less formation of these cells than in controls and consequently, lower resorption.

The small variation of the cortical thickness of allograft shows that during the study period of thirty days, the integration process occurs in the cancellous bone and not in the cortical.

The irradiation method caused the least damage to mineralization, yet results were not statistically significant. Mineralization detected in the sterilized allografts confirms the presence of bone neoformation.

Since the allografts under study relied only on their osteoinductive potential for osteogenesis, the 30-day period was considered to be too short because of the retarded osteoinduction. This is described by Chalmers³, who stresses that freeze-dried bone allografts undergo bone neoformation at a later stage, usually after a month.

When analyzing results, no significant differences were found between the sterilization methods, suggesting that they might have caused similar effects to the freeze-dried bone allografts, and therefore justify the variety of options available regarding the sterilization methods. Munting¹⁴ in his study reached a similar conclusion.

The results confirmed the findings of several authors about the possibility of clinical use of freeze-dried sterilized bone allografts^{5,7,9,12,15}. Physical changes caused by these processes must be better studied to define other more adequate parameters for the choice of the sterilization method to be utilized⁶.

Conclusion

The three sterilization methods studied caused similar effects on bone allografts regarding macroscopic and histomorphometric parameters analyzed.

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