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Estudo comparativo de três técnicas histológicas para análise da reparação óssea em tíbias de rata

Comparative study of three histological techniques to analyze the bone repair in tibias of rats

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RESUMO

O objetivo deste estudo foi validar duas técnicas adaptadas para o processamento de tecidos duros (T1-T2) sem descalcificação, comparando-as à técnica de descalcificação com EDTA a 10% no estudo da reparação óssea em tíbias de ratas. Para tanto, defeitos ósseos monocorticais foram criados em 48 ratas, que foram divididas em três grupos. O sacrifício ocorreu aos 7 e 21 dias após a cirurgia (n=8). As tíbias do primeiro grupo foram descalcificadas (E7-E21), do segundo foram submetidas à T1 com inclusão em resina poliéster (T1/7-T1/21), e do terceiro foram submetidas à T2 com inclusão gradual em metilmetacrilato (T2/7-T2/21). Análise histomorfométrica foi realizada para verificar a formação óssea no centro do defeito. A análise estatística dos resultados, por teste ANOVA dois fatores, mostrou que o tempo de sacrifício foi estatisticamente significante (p=0.01). O teste de Tukey revelou que E21 e T2/21 apresentaram médias maiores do que o T1/21. Aos 21 dias, uma maior porcentagem de ósseo neoformado pôde ser observada em relação ao período de 7 dias. Observou-se também que o fator técnica histológica (p=0,23) e sua interação com o tempo de eutanásia (p=0,09) não foram estatisticamente significantes. Os resultados sugerem que T1 revelou certas desvantagens que interferiram na qualidade e quantidade do tecido analisado. T2 foi eficiente como técnica de processamento de tecido mineralizado, permitindo a análise da reparação óssea em ambos os períodos observados sem diferenças estatisticamente significantes da técnica de descalcificação com EDTA.

PALAVRAS-CHAVE: *Osteotomia, regeneração óssea, técnica de descalcificação, técnicas histológicas.*

ABSTRACT

The purpose of this study was to validate two adapted techniques for hard tissue processing (T1-T2) without decalcification, compared to the 10% EDTA decalcification technique, while studying bone repair in rat tibia. Bone monocortical defects were created in 48 rats, which were divided into three groups. Euthanasia occurred 7 and 21 days after surgery (n=8). Tibias from the first group were decalcified (E7-E21), from the second were submitted to T1, with inclusion in polyester resin (T1/7-T1/21), and from the third were submitted to T2, with gradual inclusion in methylmetacrylate (T2/7-T2/21). Histomorphometric analysis was realized to verify the newly formed bone in the center of the defect. Statistical analysis of the results, by two factors ANOVA test, showed that the period of sacrifice was statistically significant (p=0.01). Tukey's test showed that E21 and T2/21 had higher averages than T1/21. At 21 days, a higher percentage of newly formed bone could be observed compared with the 7 days period. It could also be observed that the histological technique factor (p=0.23) and its interaction with the period of euthanasia (p=0.09) were not statistically significant. The results suggest that T1 revealed certain disadvantages which interfered in the quality and quantity of tissue analyzed. T2 was efficient as a hard tissue processing technique, allowing bone regeneration analysis for both observation periods, with no significant differences from the EDTA decalcification technique.

KEY WORDS: *Osteotomy, bone regeneration, decalcification techniques, histological techniques.*

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INTRODUCTION

The study of bone regeneration is of interest for numerous health professionals [1]. In recent years, the number of studies aimed to clarify the physiological mechanisms of bone repair in guided tissue regeneration [2,3] and reconstruction surgeries [4] has increased.

As bone is calcified tissue, it is difficult to slice it into thin sections to permit microscopic analyses. Therefore, in routine work, different techniques of tissue preparation must be used. Some of these techniques consist of the decalcification of bone specimens before further processing. Decalcification is achieved using acid solutions (formic or nitric acid) or chelating agents prior to specimen inclusion in paraffin for slicing [5].

Aside from decalcification, specific inclusions and hard tissue microtomes can also be used, which facilitate the acquisition of bone sections without prior decalcification. These methods permit diverse types of studies that were not previously possible [5].

However, the microtomes for hard tissues processing are very expensive and, therefore, not viable in many laboratories. This difficulty makes the study of hard tissues more complicated and reveals the necessity of investigating or adapting new techniques that make microscopic analyses of bone tissue possible.

Momose [6] adapted a methodology for hard tissue processing based on specimen inclusion in a polyester resin, cut in a low speed diamond saw, specimen reinclusion and surface grinding. Macedo et al. [7] used the technique proposed by Schenk et al. [8] for mineralized tissues based on gradual inclusion of the fragment in methylmetacrylate, slicing in a low speed diamond saw, followed by pressing and grinding.

The purpose of this study was to validate two techniques used to process hard tissue without decalcification (T1 and T2) and compare these with decalcification in 10% ethylenediaminetetraacetic acid (EDTA), to verify if these techniques are appropriated and can replace the use of special microtomes to evaluate the bone repair by histological and morphometrical analyses without prior decalcification.

MATERIAL AND METHODS

The study protocol was performed in compliance with the bioethical principles for animal research and approved by the Ethics in Research Committee of São José dos Campos School of Dentistry (UNESP, Brazil).

Forty-eight adult female rats (*Rattus norvegicus albinus, Wistar*) aged approximately 120 days and weighing about 400g were obtained from the Animal Laboratory of São José dos Campos School of Dentistry (UNESP, Brazil). The rats were housed in an appropriate environment and received commercial feed and water *ad libitum*.

The rats were anesthetized by intramuscular injection of 2% xylazine (Rompun; Bayer, São Paulo, Brazil; 13 mg/kg body weight) and ketamine (Francotar, Virbac, Roseira, SP, Brazil; 33 mg/kg body weight). The rats were previously weighed to confirm the correct administration dose.

After shaving the skin over the proximal area of the tibia, an incision was made and the periosteum detached. A 3 mm diameter monocortical bone defect was made in the tibia using a trephine bur under constant irrigation with saline. Muscle and skin layers were sutured and a new antisepsis procedure was performed. The rats received an intramuscular dose of 0,1 ml of antibiotic (Pentabiótico Veterinário, Fontoura Wyeth, Campinas, SP, Brazil) and an antiinflammatory (1 mg/kg - Voltaren, Novartis, São Paulo, SP, Brazil). Two experimental groups were created, containing 24 rats each one: the first group was sacrificed at 7 days and the other, 21 days after surgery, to verify if the techniques allowed the analysis in early stages of bone repair.

The tibias were removed and immediately placed in 10% formalin solution for a minimum period of two days. Each experimental group was divided in a three subgroups containing 8 rats that were submitted to decalcification with 10% EDTA (E7 and E21 groups) and to the techniques T1 (T1/7 and T1/21 groups) and T2 (T2/7 and T2/21 groups).

DECALCIFICATION (EDTA GROUPS)

After dehydration, the tibias were decalcified in a solution of 100g of EDTA (Titriplex® Merck DGaA Darmstadt, Germany), 10g of sodium hydroxide P.A. and sufficient quantity of distilled water to complete 1000ml, for about four months. Next, tibias were hemi sectioned and submitted to routine histological procedures. Each one of the two blocks of specimens were sliced in 4 semi serial 5µm thick sections (intervals of 60µm), in the center of the defect, and stained with hematoxylin-eosin (HE). For morphometrical analyses eight microscopic fields were obtained in light microscope Axiophot 2 (Carl Zeiss, Oberköchen, Germany) with original magnification of 200x, digitalized with a Sony Cybershot digital camera. The software Image-J (National Institutes of Health, for Windows) was used in order to position an integration grid with 96 points symmetrically distributed over the total area of the photograph. It was used, adding 768 points counted per rat for the point-counting planimetry. Points coinciding with bone trabeculae were individually counted. The area fraction was evaluated in percentage, using the stereology principles [9].

T1

After fixation, the specimens were washed under running water overnight, dehydrated in increasing ethanol solutions (50, 60, 70, 80, 90, and 100%) and stored in 100% ethanol for two days.

After dehydration, the tibias were submitted to T1 by inclusion in polyester resin (Orto Cristal Alpha 190 Valglass, São José dos Campos, São Paulo, Brasil) containing 1% catalyst in a rectangular silicone mould. The block was sectioned using a low speed diamond saw (Labcut® 1010, Extec Corporation®, Enfield, CT, USA) and the fragments (at least two of approximately 450µm, in the center of defect) were reincluded in the mould using the same technique. Next, the block was ground in a automatic grinder (Labpol® 8-12, Extec Corporation®, Enfield, CT, USA) using sequential water sandpapers (granulation: 400, 600 and 1200) to obtain the minimum thickness required for histological analysis (approximately 50µm), while trying to preserve the characteristics of the tissue. The material was stained by immersion in toluidine blue and analyzed under a light microscope. For morphometrical analyses two microscopic fields were obtained and the method described above was used to obtain the percentage of bone neoformation.

T2

After fixation and dehydration, the tibias were kept in xylol for 2 days for specimen diaphanization. The bone defect tissue was immersed in a resinous solution permitting the complete penetration of the resin into the specimens. A solution of methylmetacrylate (Fluka Riedel-de Haën®, Buchs SG, Switzerland) and dibutylphthalate (Fluka Riedel-de Haën®, Buchs SG, Switzerland) was prepared at a ratio of 85ml:15ml, respectively. The specimen remained in this solution for three days at room temperature. After this stage, 1g of benzoyl peroxide (Vetek Química Fina, Duque de Caxias, Rio de Janeiro Brazil) was added to the 100ml of solution and the specimen was stored for a further two days under refrigerator. Amber flasks with covers had been previously prepared for storing the specimens, containing a layer of approximately 2mm of the first solution used in this technique and 3g of benzoyl peroxide, forming a resin base. Over this base, the specimen was positioned and covered with the solution added 3g of benzoyl peroxide. This final inclusion stage lasted 2-4 days at 37°C. Once polymerization was concluded, the glass was broken.

For the following stages, 2.0 x 2.5 x 7.5mm acetate blades, a pressing device for the blades and a support for grinding the slices were constructed. The material obtained in the inclusion was cut in a low speed diamond saw (Labcut® 1010, Extec Corporation®, Enfield, CT, USA) using a 3 inch diameter diamond disc. Next, they were cleaned, glued to the acetate blade with cyanoacrylate ester (Super Bonder®, Loctite, Henkel Ltda, São Paulo, SP, Brazil) and pressed to achieve parallelism between the blade and the grinding surface. Using a support for the acetate blade, the slice was ground in a automatic grinder (Labpol® 8-12, Extec Corporation®, Enfield, CT, USA) using increasing sequential water sandpapers (granulation: 400, 600, 800, 1200 and 2500). During grinding, the slices were observed under a light microscope until the desired thickness was achieved (approximately 50 µm). Finally, the material was stained by immersion in toluidine blue and submitted to morphometrical analysis. For morphometrical analyses two microscopic fields were obtained and the method described above was used to obtain the percentage of bone neoformation.

Data were submitted to statistical analysis (ANOVA and Tukey´s test, 5% significance).

RESULTS

The figure 1 is a table that represents the histomorphometric means of trabecular bone in the analyzed region, in percentage. The histological aspects were similar between the different groups, presenting greater differences between the observation periods of 7 and 21 days (figure 1).

Statistics	Techniques					
	EDTA		T1		T2	
	7 days	21 days	7 days	21 days	7 days	21 days
n*	8	8	8	8	8	8
Mean	26,46	42,96	28,48	32,79	26,95	40,30
Standard Deviation	3,45	11,06	6,55	6,48	6,65	7,49

*n = sample´s number

Figure 1: Histomorphometric analysis: means of trabecular bone and standard deviation of the groups

The bone defect region of the rats sacrificed at 7 days was easily located and distinguishable from the cortical bone (Figure 2,3). The edges were well defined and intense bone neoformation was observed originating at the edge and growing toward the center of the defect. Bone neoformation was constituted by numerous immature and disorganized trabeculae (Figure 3).

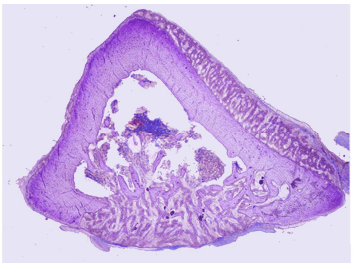

Figure 2: Bone defect region at 7 days presenting well defined edges and intense bone neoformation. Panoramic view (25x), T2, Toluidine blue

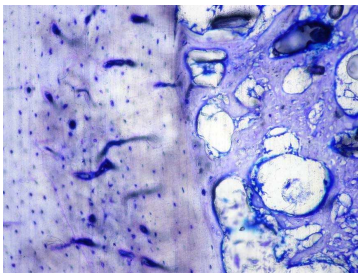

Figure 3: Limit between preexistent cortical bone and newly formed trabecular bone. 32x, T1, Toluidine blue

At 21 days, the most remarkable characteristic in all the techniques was the presence of a compact, mature and organized osseous band that joined the edges of the defect, sometimes poorly distinguished (Figure 4,5,6).

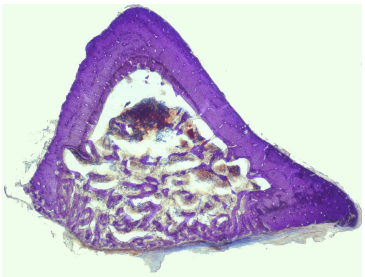

Figure 4: Organized band that joined the edges of the defect (21 days). Panoramic view (25x), T2, Toluidine blue

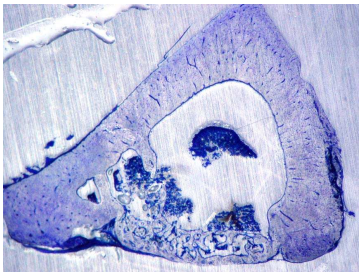

Figure 5: Organized band that joined the edges of the defect (21 days). Panoramic view (25x), T1, Toluidine blue

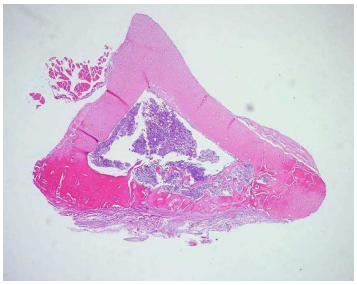

Figure 6: Bone defect region at 21 days with a organized band that joined the edges of the defect. Panoramic view (25x), EDTA, HE

Observation also revealed the presence of less bone trabeculae in the marrow canal when compared to the 7 day period. The newly formed bone trabeculae were more mature and organized, with smaller and fewer lacunae than in those of the first period (Figure 7).

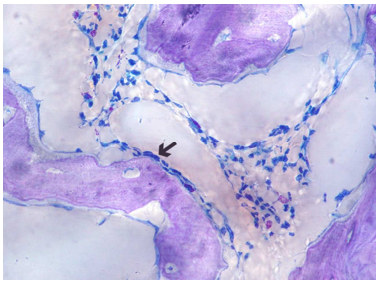

Figure 7: Newly formed bone trabeculae surrounded by cells suggestive of osteoblasts. High power view (630x), T2, Toluidine blue

The majority of the trabeculae from the interior of the medullar canal were remodeled. Osteoblasts, which were occasionally flattened, were observed arranged in line, outlining some trabeculae. Reversed basophilic lines were seen, as well as some giant multinuclear cells, confirming the bone remodeling phase and some mast cells were noted. Soft tissue was clearly observed in the decalcification technique, but it was in good conditions for histological analysis in T2.

Application of the ANOVA test showed that the sacrifice time was a statistically significant factor (p=0.0001). Thus, for the 21 day period, a higher percentage of newly formed bone (38.68±9.29) was observed when compared to the 7 day period (27.29±5.56). The effect of the interaction between the variables (technique and sacrifice time) and the effect of different techniques were not statistically significant (p=0.0963 and 0.2328, respectively). Tukey´s test (5%) was also made to verify which group had the higher or smaller averages in each period, significantly different among the groups. When the average values of the six experimental conditions studied were compared by Tukey´s test, two homogeneous groups were formed. It was established that the least favorable condition for bone repair evaluation was T1 at 21 days (T1/21), probably because this technique has some disadvantages that could influence in the results, like the bubble formations, the incomplete resin penetration in the marrow canal, the lack of grinding standardization and difficulties regarding the correct reinclusion of the slices, which were sometimes not parallel to the grinding plane.

The percentage of newly formed bone for the 7 day period was similar among all the techniques analyzed. However, for 21 days, this percentage was similar between the EDTA (42.96±11.06) and T2 techniques (40.30±7.49), but differed for T1 (32.79±6.48). No significant statistical differences were found between the other subgroups.

DISCUSSION

Among the histological procedures used to analyze hard tissue, decalcification can be cited as one of the main techniques. One of the most commonly used decalcification agents is EDTA. Many researchers have used this agent at different concentrations [10,11] and achieved excellent preservation of the tissue, making the use of immunohistochemistry possible. However, this substance presents certain inconveniences, such as the time required for complete calcium salt removal. Despite the fact that formic acid, another decalcification agent commonly used in bone repair studies, be faster than EDTA, the disadvantage of its use is the difficult of preserve antigen for immunohistochemistry [12,13,14].

Other techniques exist in which bone slices without prior decalcification are used. For hard tissue histological preparation, a specific microtome is currently used to achieve slices without prior decalcification of the material. After fixation and dehydration, the tissue is included in resin forming blocks that provide sufficient support to obtain slices of the desired thickness. After acquiring these slices, the material is stained and analyzed under a light microscope [15,16,17].

However, due to high cost of such microtomes, the development of alternative techniques for the evaluation of hard tissue that preserve the mineral structural and provide rapid processing is under investigation.

The purpose of the present study was to compare two techniques for hard tissue analysis without prior decalcification and compare these with 10% EDTA decalcification, which is the gold standard. According to the results, T1 did not differ from the other techniques in the initial period, although it presents unfavorable conditions that may influence the research. Certain difficulties were found, such as bubbles, incomplete resin penetration in the marrow canal, the lack of grinding standardization and difficulties regarding the correct reinclusion of the slices, which were sometimes not parallel to the grinding plane that could influence the histomorphometric analysis and results, especially in the 21 days sacrifice period. This last disadvantage was also observed by Costa Filho et al. [18].

It was important to observe two experimental periods of bone repair. Only the later period of repair results in statistical difference between resin techniques (polyester T1, methylmetacrylate T2) and EDTA. Perhaps, there is an influence of maturation of bone trabeculae in impregnation process of the specimens in resin T1. It is possible that a variable diffusion of this resin, with high viscosity, in mature bone trabeculae, instead nearly formed bone, increases bubbles and incomplete resin penetration, modifying the histomorphometric analysis in T1/21.

The bone sections embedded in methyl methacrylate can also provide a good option for study the immunohistochemistry of undecalcified tissue. Gomes et al. [19] modified the technique using a quick decalcification because of potential destruction of tissue antigenicity by highly exothermic polymerization. Its results suggested that this method is easy, fast, and effective to perform both histomorphometry and immunohistochemistry in the same bone fragment. In 2009, Torgersen et al. [20] investigated in non-decalcified vertebra sections of Atlantic salmon, embedded in methyl methacrylate, the col1a1 and col2a1 expression by in situ hybridization. They concluded that this resin offers easy preparation of large and problematic tissues and possibility of carrying out both immunohistochemistry and in situ hybridization analyses using standard protocols.

Bubbles formation, the partial penetration of the resin used in this study and specimen reinclusion difficulties were due to the high viscosity of the resin. The grinding in T1 was performed by manual handling of the resin blocks. Therefore, this stage was susceptible to variations according to the pressure applied against the sandpaper, which was not uniform throughout the surface of the resin block.

To account for the difficulties found and based on Macedo et al. [7], modification of the processing of mineralized tissue without decalcification was proposed. Thus, after fixation, the tibiae were dehydrated, diaphanizated and gradually included in methylmetacrylate solution with the aim of reducing bubbles and promoting resin penetration into the bone tissue and marrow canal.

Next, a press device was used to obtain a thin uniform layer of glue. In the following stage, a support for grinding was used permitting a greater parallelism between the grinding surface and the blade uniform grinding of the slice.

However, some inconveniences were still observed, like resin contraction, which was high during polymerization, requiring the insertion of large quantities of the resinous solution at final stage of inclusion.

T2 was efficient for tissue processing, allowing the analysis of bone repair for both periods studied, in contrast to T1, which showed certain disadvantages that could complicate and influence the histomorphometric analysis. According to Troiano et al. [21] histological evaluation is a complex, multistep process culminating in tissue staining. All of the steps leading up to the staining affect the final quality, but too often the effects of these preparations are not given enough consideration. The treatment and preservation of tissue specimens can have a marked impact on results.

CONCLUSION

It was concluded that T2 allowed adequate hard tissue preservation, providing ideal conditions for qualitative and quantitative bone repair analyses replacing the use of special microtomes.

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