RADIOISOTOPIC EVALUATION OF BONE REPAIR AFTER EXPERIMENTAL SURGICAL TRAUMA

AVALIAÇÃO RADIOFARMACOLÓGICA DO REPARO ÓSSEO APÓS TRAUMA CIRÚRGICO PADRONIZADO

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BACKGROUND: Scientific approach of the bone reaction after surgical procedures provides valuable information on methods and techniques. The purpose of this study was to follow this process using a radioisotope marker of bone remodelling. MATERIAL AND METHODS: Two bone cavities were created (one for every tibia) in adult Wistar male rats using a 0.5 mm spherical burr; left tibial cavities were filled with bovine freeze-dried bone; the right ones were left unfilled for control. Scintigrams were done with sodium methylene diphosphonate (MDP) labelled with radioactive pertechnetate ($^{99m}TcO_4^-$) to evaluate the inflammatory response and the local osteoblastic activity. The evolution of bone repair was additionally evaluated by light microscopy. RESULTS: Our results have shown that the highest bone activity was recorded between the 7th and the 14th day after surgery. The morphological analysis confirmed the results obtained with radioisotope analysis and did not reveal significant differences regarding the evolution of bone repair between the filled and the unfilled defects. CONCLUSION: We confirmed that ^{99m}Tc - MDP is a valuable tool to study bone repair, as it was able to show subtle alterations of bone activity even in lesions as small as those created herein (0.5 mm wide, 0.5 mm deep).

UNITERMS: Bone repair; Scintigraphy; Light microscopy; Freeze-dried bone; Surgical trauma; Rat.

INTRODUCTION

There is a vast literature about the process of bone repair, and much of it deals with biomaterials which have been developed in an attempt to accelerate that process and to avoid donor site morbidity. The first studies on bone repair and bone substitutes are dated back to the beginning of the 20th century [8,27].

Despite the enormous evolution in the scientific approach of biological questions, information given by conventional clinical methods of assessing healing response is rather fragmentary. Radiographic exams, for instance, do not show the changes occurring at the very beginning of the process; the clinical manifestations not only take time to be observed, but also are highly influenced by subjective factors like mood and others.

By contrast, bone scintigraphy with ^{99m}Tc–labeled phosphonates has several advantages as a diagnostic method, being capable of revealing even subtle changes of bone activity at the early stages of the healing response, thus providing information on graft viability. This is so because it takes advantage of the strong affinity of biophosphonates to metabolically active bone, as it occurs in inflammation and other conditions [17,15]. Besides, the sensivity of this method to

detect bone alterations is by far higher than conventional or panoramic X-ray exams [13,10]. Bone uptake of the ^{99m}Tc complexes varied from 40-60% at 30 min postinjection and the uptake in soft tissue is minimum [4]. The uptake of radioactive material depends on the regional blood supply and on the degree of metabolic activity of the newly forming bone, and behaves as a probe of osteogenic activity [2,20,21]. Radionuclide bone imaging may accurately monitor the revascularization and bone regeneration after the bone graft implantation [11]. The uptake of this radiopharmaceutical provides an opportunity to evaluate bone activity and can be a potential research tool, especially in animal models.

Many biomaterials exist which have been useful to stimulate the bone repair of surgically-created defects and occasionally alleviate discomfort and pain related to the first post-surgical days [24]. Freeze-dried bone is one of those materials, and it has been indicated in a number of clinical situations as for the filling of large cystic/tumoral cavities or of those alveolar sockets resulting from the removal of included/impacted teeth [7]. The effectiveness of freeze-dried bone to reduce the time required for bone repair as compared to autogenous bone grafts was reported in the early 50s [16, 22]. Although it has been stated [9] that freeze-dried bone has the potential to function physically as a nidus for the growth of appositional new bone in alveolar sockets following tooth removal, clinical reports demonstrated that the filling of surgical defects with this material does not alter the evolution of the local inflammatory reaction [7,14].

In the present investigation we studied the efficacies of the filling of surgically created defects in rat tibiae with freeze-dried bovine bone. This was done by following the regional uptake of ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP). Radionuclide countings were coupled with histological analysis in order to evaluate the effectiveness of ^{99m}Tc-MDP uptake as a reliable marker of bone remodelling.

MATERIALS AND METHODS

Animals and treatment

72 adult, male Wistar rats (180-220 g) were obtained from animal facilities of the Institute of Biomedical Sciences, University of Sao Paulo. The NIH guidelines for the care and use of laboratory animals have been observed; the experimental design was approved by the Ethics Committee, Faculty of Medicine, University of São Paulo[6]. Chloral hydrate was used as the general anesthetic agent (400 mg/kg, ip) and standard aseptic techniques were applied. Round unicortical defects were created in the metaphysis of both tibiae according to Virolainen, et al.[30] (1997), except that a 0.5 mm cylindrical burr was used, avoiding invasion of the subjacent bone marrow (Figure 1A). The defects on the left tibiae were filled with freeze-dried bone granules (Osteon®, Cirumedica, Cotia, SP), moisted with sterile phosphate-buffered saline (PBS) immediately prior to use. The defects of the right tibiae were left unfilled, for control. The soft tissues were sutured in two layers with silk thread.

Radiopharmaceutical injection and sample collection

The animals were sacrificed at 1, 3, 7, 14, 21 or 28 days after surgery. Two hours before death under deep ether anesthesia, 500 µCi (18.5 MBq) of ^{99m}Tc-MDP (Group 1, n = 36) or of $Na^{99m}TcO_4$ (Group 2, n = 36) were injected intravenously in 0.2 ml saline. The tibiae were dissected free from any adherent tissue and two segments were cut out, using a steel disc: one of them contained the defect and the other one was a contiguous, proximally adjacent segment, without defect. These segments were then labeled as follows: Lof = left, operated, filled; Lcc = left, contiguous, control; Rou = right, operated, unfilled; Rcc = right, contiguous, control. One segment of every

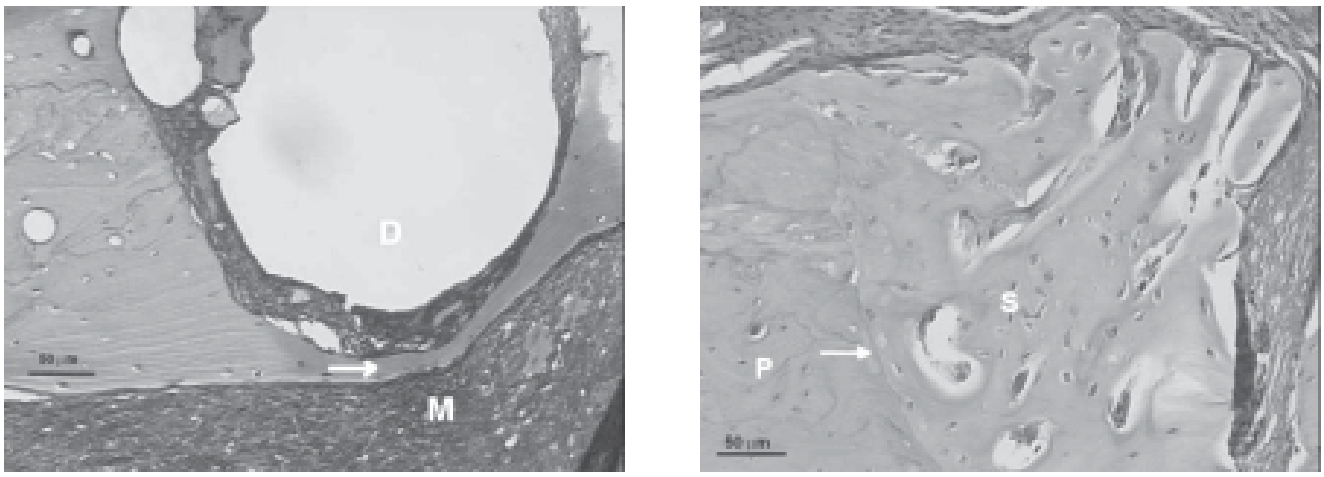

FIGURE 1- Photomicrographs of the surgically-created (unfilled) defects in rat tibia. In **A**, 1 day after surgery. The arrow indicates a thin layer of cortical bone between the bottom of the defect (**D**) and the bone marrow (**M**). In **B**, 21 days after surgery. The arrow points a divisory line between the newly formed bone (primary bone, **P**) and the old, normal bone (secondary bone, **S**). Haematoxylin-eosin staining.

femur was also cut out, at a middle position, and studied as a naïve control.

Radioactivity countings and histological analysis

All samples were weighed and immediately immersed in 2.0 ml of 10 % formaldehyde solution (operated segments) or distilled water (intact segments). Radioactivity was determined in a Packard Cobra-II® gamma counter and expressed as counts/min (cpm) per mg of fresh weight. The samples from group 1 animals (injected with ^{99m}Tc-MDP) were fixed in 10% formalin, decalcified in 60% formic acid, dehydrated and embedded in Paraplast®. Serial 7-µm thick sections were stained with haematoxylin-eosin for light microscopy examination. Pictures were taken with a CCD® camera (MTI) using a NIH Image software and processed in a Power Macintosh computer using Adobe Photoshop® software.

Statistical analysis

Results were analysed by one-way analysis of variance and the Tukey-Kramer multiple comparisons test. A 2.01 version Graph Pad In stat™ software was used for this purpose. When appropriate, the Student's t test was also used. In curves describing the ratios of ^{99m}Tc-MDP uptake (see Figure 6), the points in the segments comprising 14–28 post-operative days were fitted by linear regression, and the angular coefficients were then compared by analysis of variance.

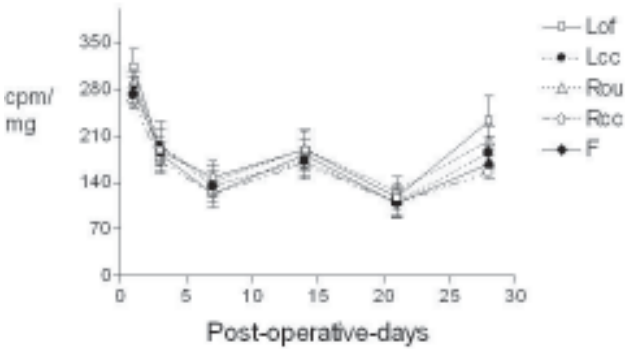

FIGURE 2- Uptake of $Na^{99m}TcO_4$ in tibiae and femurs of rats. The tracer (*ca.* 500 µCi) was injected i.v. and the animals were sacrificed 2 h later. Radioactivity (in cpm) was counted in fragments of tibiae which included the surgical defect and in contiguous, defect-free segments. A distant, naïve bone fragment (femur) was also counted, for control. Left tibial defects were filled with freeze-dried bone; the right ones were left unfilled, and groups of animals (n = 6 in every group) were sacrificed at the indicated times after surgery. Abbreviations: Lof = left, operated, filled; Lcc = left, contiguous, control; Rou = right, operated, unfilled; Rcc = right, contiguous, control; F = femur. Values are mean ± SEM (cpm/mg wet weight)

RESULTS

Since $Na^{99m}TcO_4$ cannot bind directly to any ligand [23], the results obtained with its i.v. injection give information about the regional blood flow. Accordingly, Figure 2 shows that blood flow within bone was essentially similar in all segments in all post-operative (P.O.) days. A slight, yet significantly enhanced flow (about +67%) was detected in the 1st P.O. day, an effect seen not only in the operated segments (Lof, Rou) but also in the contiguous, defect-free controls (Lcc, Rcc).

The affinity of MDP towards bone can be clearly seen in Figure 3, which shows that, in femur, countings of ^{99m}Tc-MDP are ca. 1,500% higher ($P<0.001$) than those of $Na^{99m}TcO_4$ at the 3rd P.O. day. However, we observed a significant, progressive fall of that difference, and at the 28th day it was reduced to ca. 730% ($P<0.001$).

The uptake of ^{99m}Tc-MDP in defect-bearing and in defect-free tibial segments is compared in Figure 4. The operated fragments bound the MDP tracer significantly more than did the contiguous fragments. At the 7th P.O. day the difference reached ca. 57%, and this progressively felt down to 34% at the 28th day. Essentially undistinguishable results were observed in fragments containing the defects filled with freeze-dried bone and in their contiguous, control counterparts (Figure 5). At the 7th P.O. day the difference of radiotracer uptake between operated and non-operated areas was about 51% and at the 28th day it was about 42%. On the other hand, no remarkable differences were detected when the ratios of MDP uptake in filled defects were compared to those in unfilled cavities, being both situations corrected as a function of the local blood flow (Figure 6).

Notwithstanding, it is interesting to notice that not only there is an obvious exacerbation of ^{99m}Tc-MDP uptake around the 14th P.O. day, but also the slope of the 'recovery' part of the curve (from the 14th up to the 28th day) is slightly faster for the unfilled cavities (Rou/Rcc, dashed line) than for the filled ones

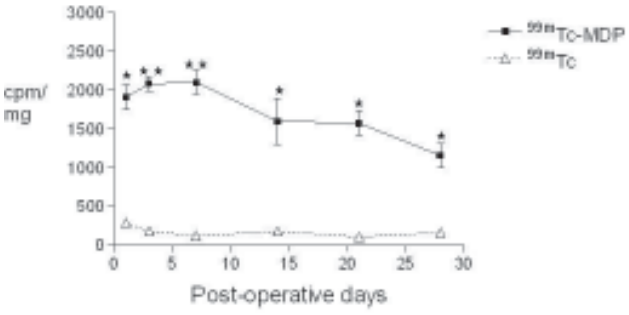

FIGURE 3- Uptake of ^{99m}Tc-MDP and $Na^{99m}TcO_4$ in femurs of rats. The tracers (*ca.* 500 µCi) were injected i.v. in parallel groups of animals which were sacrificed 2 h later. Values are mean ± SEM (cpm/mg wet weight) of data from 6 animals for every group. Asterisks indicate significant differences regarding the corresponding points of $Na^{99m}TcO_4$ uptake (*$P<0.05$; **$P<0.01$)

(Lof/Lcc, solid line).

Light microscopy examination of our material did not reveal significant differences of the bone repair process regarding the presence or absence of foreign material in the osseous cavities. Figure 1B shows, at the 21st P.O. day, the almost completely recovered defect in an unfilled cavity. The pictures seen with filled cavities (not shown) were essentially identical.

DISCUSSION

Surgically-created defects in rat tibia constitute a good model for studies on bone repair for such reasons as easy surgical access, convenient bone cortical thickness, great volume of bone marrow, and others [1,26,28,30].

In the experimental model used herein, the defect dimensions were small enough to allow proper bone regeneration. Being so, the cavity could not be classified as a 'critical bone defect', i.e. an intra-osseous wound with poor healing evolution [3]. In fact, the 0.5 mm depth of the cavity avoided invading the underlying bone marrow (Figure 1A) and thus, in the filled defects ('Lof', see legend to Figure 2), it was assured that the biomaterial stayed inside cavity, without being expelled out as it would be the case if the blood flowed from bone marrow [3,26,28,30].

The observed increases in bone blood flow in the reference region (femur, F) might not be due to some direct, local reaction following the surgical act, but would be rather consequent to a regional vascular effect, triggered by the surgical procedure. In fact, there are several peculiarities about blood supply in mineralized tissues which are presently far from being completely understood [12,29].

It is well established that diphosphonates avidly bind to hydroxylapatite and can thus cause various cellular effects in bone cells[25]. Although the exact explanation for this phenomenon is at present unresolved, it is presumable that the fading of the aforementioned 'vascular effect' triggered at bone level (even in distant bones) by the surgery may play a role.

The uptake in the operated segments shows that the tracer was actively uptaken by surgically-stimulated bone around

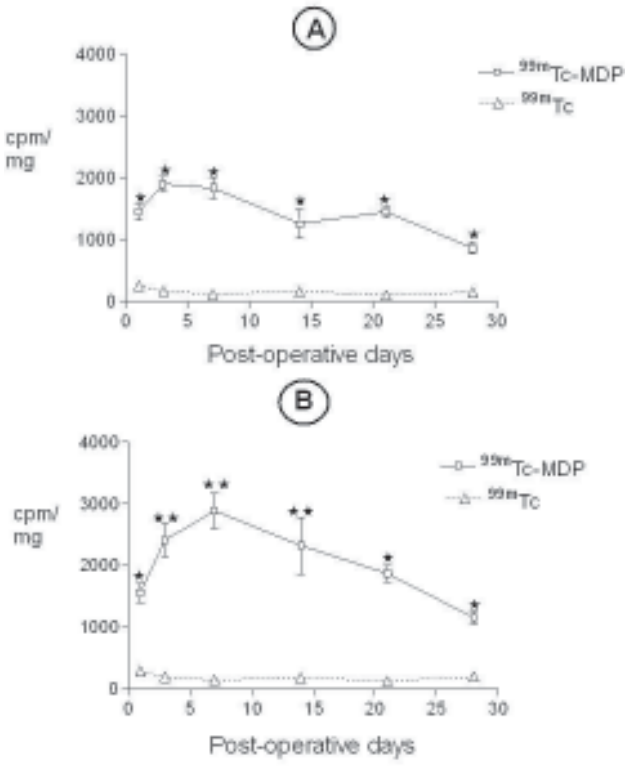

FIGURE 4- Uptake of ^{99m}Tc-MDP and Na$^{99m}TcO_4$ in right tibiae of rats. The tracers (*ca.* 500 μCi) were injected i.v. in parallel groups of animals which were sacrificed 2 h later. Radioactivity (in cpm) was counted in defect-free segments (control, panel A) and in segments containing unfilled surgical defects (panel B). Values are mean ± SEM (cpm/mg wet weight) of data from 6 animals for every group. Asterisks indicate significant differences regarding the corresponding points of Na$^{99m}TcO_4$ uptake (*P<0.05; **P<0.01)

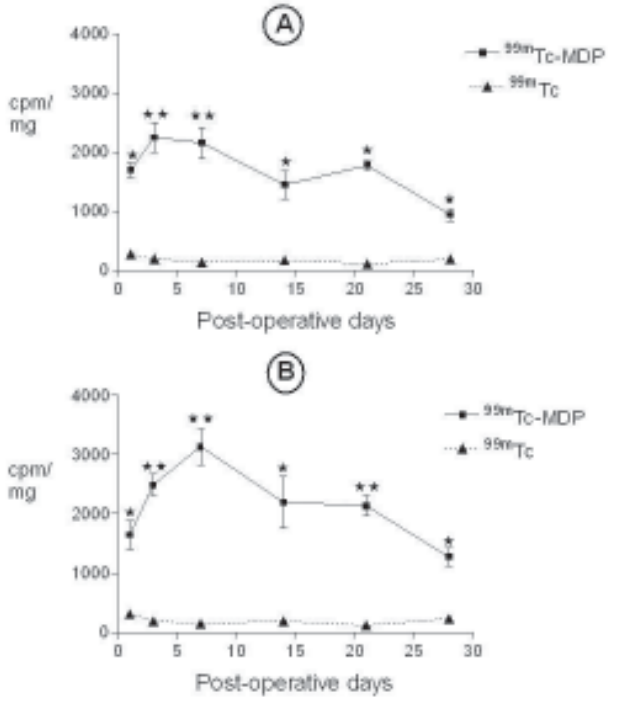

FIGURE 5- Uptake of ^{99m}Tc-MDP and Na$^{99m}TcO_4$ in left tibiae of rats. The tracers (*ca.* 500 μCi) were injected i.v. in parallel groups of animals which were sacrificed 2 h later. Radioactivity (in cpm) was counted in defect-free segments (control, panel A) and in segments containing the surgical defects filled with freeze-dried bone (panel B). Values are mean ± SEM (cpm/mg wet weight) of data from 6 animals for every group. Asterisks indicate significant differences regarding the corresponding points of Na$^{99m}TcO_4$ uptake (*P<0.05; **P<0.01)

the 7th day after surgery, and this occurred at a time point when cellular activity and organic matrix production conceivably were at their maximum levels[17,18,19-30]. In addition, it is known that increased osteoblastic activity results in an increased deposition of the radiopharmaceutical into the affected area in comparison to the nearby or contralateral normal osseous tissues[20,21].

As far as MDP uptake is concerned, the filling of osseous defects with freeze-dried bone did not interfere on the time course of the bone healing evolution. In other words, it seems likely that even if the filling of a surgically-created bone cavity with a freeze-dried bone preparation may conveniently elicit osteoconduction, the elapsed time for tissue healing may not be shortened or the clinical symptoms alleviated. Our findings confirm that bovine osseous grafts do not interfere with bone repair nor do they elicit ortotopical bone formation when the dimensions of the defect are not critical. Concerning the light microscopy, similar results have also been reported in other experimental approaches in the literature[5].

CONCLUSIONS

In conclusion, our data support the view that the measurement of the uptake of ^{99m}Tc-MDP can provide valuable information on the evolution of bone reaction after graft procedures and be used as a research tool in animal models.

RESUMO

Este trabalho objetivou estudar a evolução temporal do processo de reparo ósseo em tíbia de rato, após trauma cirúrgico padronizado. A incorporação do radiofármaco ^{99m}Tc-MDP na região afetada foi tomada como medida indireta da intensidade de reação tecidual; foi feito também acompanhamento histológico do processo de reparo. Foram realizadas cirurgias nas duas tíbias de 72 animais divididos em 2 grupos, sendo sacrificados em diferentes dias pós-operatórios (1, 3, 7, 14, 21 e 28 dias p.o.). As cavidades criadas nas tíbias esquerdas foram preenchidas com osso liofilizado bovino, e as direitas serviram como controle (não preenchidas). Grupos paralelos de animais foram injetados com ^{99m}Tc para avaliar a influência do fluxo sangüíneo regional nos resultados. Duas horas após a injeção dos radiofármacos os animais foram sacrificados, a radiatividade foi contada tanto nos fragmentos das tíbias contendo os defeitos cirúrgicos como em fragmentos intactos de fêmur e de tíbias, como controle. Os resultados indicam que a maior atividade do tecido ósseo ocorreu entre 7 e 14 dias p.o. O emprego do radiofármaco mostrou ser de valor na avaliação do reparo dada sua sensibilidade. Não houve efeito significativo da presença de osso liofilizado sobre a evolução do reparo ósseo.

UNITERMOS: Reparação óssea; Radiofármaco; Microscopia óptica; Osso liofilizado; Trauma cirúrgico; Ratos.

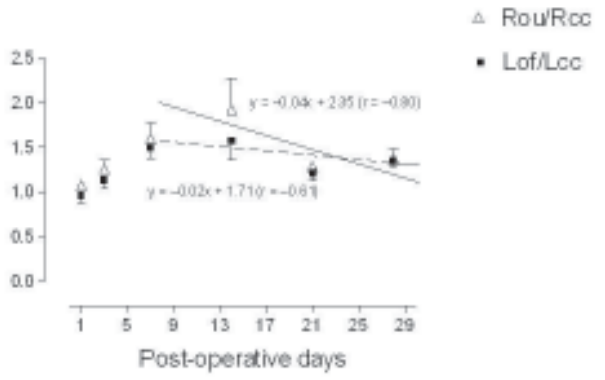

FIGURE 6- Ratios of ^{99m}Tc-MDP uptake in tibiae of rats. The straight lines represent the linear regression calculations of the descending part of the curves. Dashed line refers to right tibial (with unfilled defects) values; solid line refers to the left tibial (with defects filled with freeze-dried bone) values. The tracer (*ca.* 500 mCi) was injected i.v. and the animals were sacrificed 2 h later. Abbreviations: see legend to Fig. 2. Values are mean ± SEM

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REFERENCES

1- Alberius P, Gordh M, Lindberg L, Johnell O. Effect of cortical perforations of both graft and host bed on onlay incorporation to the rat skull. Eur J Oral Sci 1996; 104:554–61.

2- Alexander JM. Radionuclide bone scanning in the diagnosis of lesions of the maxillofacial region. J Oral Surg 1976; 34:249–56.

3- Bach DE, Burguess LPA, Zislis T, Quigley N, Hollinger JO. Cranial, iliac and demineralized bone freeze-dried bone grafts of the mandible in dogs. Arch Otolaryngol Head Neck Surg 1991; 117:390–5.

4- Banerjee S, Samuel G, Kothari K, Unni PR, Sarma HD, Pillai MR. Tc-99m and Re-186 complexes of tetraphosphonate ligands and their biodistribution pattern in animal models. Nucl Med Biol. 2001 Feb; 28(2): 205-13.

5- Batista PS, Sant'Ana Filho M. Microscopic evaluation of healing in osseous cavities submitted to implants of lyophilized bovine bone (Bio-Oss) in femur of female rats. Rev Pos-Graduacao Fac Odontol Univ S Paulo 2001; 8:62–9.

6- Bayne K. Developing guidelines on the care and use of animals. Ann N Y Acad Sci 1998; 30:105–10.

7- Becker N, Urist M, Becker BE, Jackson W, Parry DA, Bartold M, Vicenzzi G, De Georges D, Niederwanger M. Clinical and histological observations of sites implanted with intraoral autogenous bone grafts or allografts: 15 human case reports. J Periodontol 1996; 67:1021–33.

8- Brown WL, Brown CP. Preliminary report on experimental bone and periosteal transplantation. Surg Gynecol Obstet 1913; 27:681–9.

9- Brugnami F, Then PR, Moroi H, Leone CW. Histologic evaluation of human extraction sockets treated with demineralized freeze-dried bone allograft (DFDBA) and cell occlusive membrane. J Periodontol 1996; 67:821–5.

10- Bush FM, Harrington WG, Harkins SW. Interexaminer comparison of bone scintigraphy and panoramic radiography of temporomandibular joints: correlation with signs and symptoms. J Prosthet Dent 1992; 67:246–51.

11- Chen B, Pei GX, Wang K, Fan YX, Wang H, Jin D, Wei KH. Repair of tibial defect with tissue-engineered bone graft and radionuclide bone imaging in goats. Di Yi Jun Yi Da Xue Xue Bao 2002 ;22(11):966-9.

12- Clemens TL. Vasoactive agents and bone metabolism. In: Bilezekian JP, Raisz LG, Rodan GA (editors). Principles of Bone Biology. San Diego: Academic Press, 1996. p.597–605.

13- Craemer TD, Ficara AJ. The value of the nuclear medical scans in the diagnosis of temporomandibular joint disease. J Oral Surg 1986; 58:382–5.

14- Crivello Jr O, Lara PF, Oliveira-Filho RM, Menecheli-José AP, Galantier, C. Avaliação cintigráfica da reparação alveolar pós-cirúrgica com osso liofilizado. In: Proceedings of the 14ª. Reunião da Sociedade Brasileira de Pesquisas Odontológicas 1997; p. 162.

15- Cronhjort M, Sääf M, Sjönberg HE, Schnell PO, Jacobsson H. Influence of the phosphate balance on the activity distribution of ^{99m}Tc-hydroxy-methylene diphosphonate. Experimental studies in the mouse. Acta Radiol 1998; 39:427–33.

16- Dubau R, Meunier JP, Demarty R, Henaff F. Une nouvelle méthode de conservation des greffons osseux par dessiccation sous vide à partir de l'élat congelé (lyophilisation). Presse Méd 1952; 60:1402.

17- Garcia DA, Jansons D, Kapur KK. Bone-imaging and semiconductor probe measurements of technetium-99m-polyphosphate in the detection of periapical pathology in dog. Arch Oral Biol 1976; 21:167–74.

18- Genant HK, Bautovich GJ, Singh M, Lathrop KA, Harper PV. Bone-seeking radionuclides: an in vivo study of factors affecting skeletal uptake. Radiology 1974; 113:373–82.

19- Hutchinson IL, Cullum ID, Langford JA, Jarritt PH, Ell PJ, Harris M. The investigation of osteoradionecrosis of the mandible by 99mTc-methylene diphosphonate radionuclide bone scans. Br J Oral Maxillofac Surg 1990; 28:143–9.

20- Katzberg RW, O'Mara RE, Tallents RH, Weber DA. Radionuclide skeletal imaging and single photon emission computed tomography in suspected internal derangement of the temporomandibular joint. J Oral Maxillofac Surg 1984; 42:782–7.

21- Kelly JF, Cagle JD, Stevenson JS, Adler GJ. Technetium-99m radionuclide bone imaging for evaluating mandibular osseous allografts. J Oral Surg 1975; 33:11–7.

22- Kreuz FP, Hyatt GW, Turner TC, Bassett AL. The preservation and clinical use of freeze-dried bone. J Bone Joint Surg 1951; 33A:863–72.

23- Lever SZ. Technetium and rhenium compounds. In: Wagner Jr HN (editor). Principles of Nuclear Medicine. Philadelphia: Saunders 2nd ed 1995. p.213–20.

24- Lewandrowski KU, Gresser JD, Wise DL, Trantolo DJ. Bioresorbable bone graft substitutes of different osteoconductivities: a histologic evaluation of osteointegration of poly(propylene glycol-co-fumaric acid)-based cement implants in rats. Biomaterials 2000, 21(8):757-64.

25- Miller SC, Jee WSS. The effect of dichloromethylene diphosphonate, a pyrophosphate analog, on bone and bone cell structure in the growing rat. Anat Rec 1979; 193:439–62.

26- Okamoto T, Garcia Jr IR, Magro-Filho O, Storti SC. Implante de osso anorgânico em cavidade óssea: estudo histológico em ratos. Rev Odontol UNESP 1994; 23:213–4.

27- Phemister DB. The fate of transplanted bone and regenerative power of its various constituents. Surg Gynecol Obstet 1914; 19:303–33.

28- Rodriguez y Baena R, Zaffe D, Brusotti C, Marchetti C, Botticelli A, Rizzo S. Materiali osteoconduttori: sperimentazione animali e analisi strumentali II. Minerva Stomatol 1997; 46:635–47.

29- Solheim E, Pinholt EM, Talsnes O, Larsen TB, Kirkeby OJ. The relationship between revascularisation and osteogenesis in fresh or demineralised bone grafts. Eur Surg Res 2001; 33:42–6.

30- Virolainen P, Elima K, Metsäranta M, Aro HT, Vuorio E. Incorporation of cortical bone allografts and autografts in rats. Acta Orthop Scand 1998; 69:537–44.

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