Histol Histopathol (2001) 16: 481-485

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Prolonged immobilization-induced stress delays alveolar bone healing. A histometric study in rats

K.F. Bombonato Prado, T.L. Lamano Carvalho, J.A.A. Franci and L.G. Brentegani

Department of Morphology, Stomatology and Physiology, Dental School of Ribeirão Preto, University of São Paulo, Brasil

Summary. The purpose of the present study was to investigate the effect of prolonged immobilization-induced stress on reparative bone formation, using the rat alveolar healing as an experimental protocol. Stress was attained by immobilization for 2 hours a day, beginning three days before extraction of the upper right incisors and continuing until sacrifice. The stress condition was assayed on the basis of plasma corticosterone concentration (measured by double-antibody radioimmunoassay), which increased by 2.5 to 4 times in rats submitted to immobilization. The volume density of neoformed bone filling the alveolar socket was quantified by a histometric differential point-counting method 7 to 21 days following tooth extraction. Stress caused a significant delay in reparative bone increment, somewhat related to impairment of coagulum remission and/or organization.

Key words: Stress, Bone healing, Alveolar healing

Introduction

Bone metabolism is controlled by a number of hormones (Bilezikian et al., 1996a,b), among them the glucocorticoids. While physiological concentrations of adrenal glucocorticoid (cortisol in humans, corticosterone in rats) are essential for bone cell differentiation and function (Lukert and Kream, 1996), pharmacological doses, introduced for therapeutic use because of their potent anti-inflammatory and immunosuppressive effects, cause loss of bone mass and pathological fractures (Munck and Guyre, 1989; Lukert and Kream, 1996; Webster et al., 1998).

Both physical and psychological stress may activate the hypothalamus-pituitary-adrenal axis causing a cascade of increased secretions of corticotrophin-releasing hormone (CRH), adrenocorticotropin (ACTH) and glucocorticoids (Elenkov et al., 1999). Short- or long-term immobilization may be stressful to laboratory animals, the magnitude of the response depending on the intensity and duration of the stimulus. An experimental protocol of prolonged intermittent immobilization has been used in our laboratory and has been confirmed as an aversive stimulus for rats, resulting in increased plasma ACTH and corticosterone concentrations (Almeida et al., 1998a). The purpose of the present study was to investigate whether prolonged immobilization-induced stress interferes with reparative bone formation, using as experimental model the alveolar healing, i.e., the progressive filling of tooth extraction socket with neoformed bone.

Offprint requests to: Dr. Teresa L. Lamano Carvalho, Faculdade de Odontologia de Ribeirão Preto - USP, Av. do Café s/n, 14040-904 Ribeirão Preto, SP, Brasil. e-mail: tllc@forp.usp.br

Material and methods

Male Wistar rats (180g mean body weight) were used. The animals were housed four to a cage in plastic boxes (40x32x17cm) in a noiseless room, under controlled lighting (lights on from 6.00 to 18.00) and environmental temperature (23±2 °C). Laboratory chow and tap water were given *ad libitum*.

Stress was attained by immobilization inside plastic tubes dimensioned to produce stress without promoting unnecessary pain (4.5 cm in diameter x 15.5 cm long), for 2 hours a day (starting at 9.00 am), beginning three days before tooth extraction and continuing until sacrifice. Undesirable stress was avoided as much as possible by gentle handling and noiselessness throughout the experiment. Control animals were left undisturbed in their cages.

Tooth extraction

The rats were anesthetized with an intraperitoneal injection (25 mg/100g body weight) of 2,2,2-tribromoethanol (Aldrich, USA) and the upper right incisors were extracted with forceps after disconnection of the surrounding gingiva, as previously described (Lamano Carvalho et al., 1997a). Immediately after surgery, the gingival tissues were sutured and a single intramuscular dose of antibiotic (Pentabiótico Veterinário, Wyeth, São Bernardo do Campo, SP, Brasil,

0.2 ml/rat) was administered. All the procedures were conducted in accordance with ethical and humane principles of animal research, as approved by institutional guidelines.

Control (n=21) and stressed (n=21) rats were killed by decapitation 7, 14 and 21 days following tooth extraction, in the morning after the last stressing session. Trunk blood was collected into heparinized tubes and plasma was separated by centrifugation and stored at -70 °C for corticosterone measurement by double-antibody radioimmunoassay, using a commercial kit (Coat-A-Count Rat Corticosterone, Diagnostic Products Corporation, DPC, Los Angeles, United States). All samples were measured in a single assay.

Immediately after blood collection, the mandibles were removed and the heads were immersed in 10% formalin for 48 h. After fixation, the maxillae were dissected and divided along the median sagittal plane and the right halves were cut tangentially to the distal surface of the molars, decalcified and processed for paraffin embedding. Semi-serial longitudinal 6 μm-thick sections cut at 60 μm intervals were stained with haematoxylin and eosin. An integration eyepiece with 25 equidistant points fitted to a light microscope was used to estimate the volume fraction of healing alveolar components by a differential point-counting method, as previously described (Lamano Carvalho et al., 1997a). A total of 1,500 points were counted from the apical to the cervical areas of each alveolus (final magnification x420), by the same observer blinded for the sample group, the percentage of points lying on connective tissue, coagulum and bone trabeculae being proportional to their volume density.

The results were analyzed statistically by the non-parametric Kruskal-Wallis test (GMC Basic Software).

Results

Plasma corticosterone concentrations increased by 2.5- to 4-times in rats submitted to prolonged intermittent immobilization (Fig. 1).

The sequential reparative changes were recognized by histological examination in the alveolar socket of control and stressed rats 7 to 21 days after tooth extraction. By the 7th day, coagulum remnants were noticed in addition to newly formed bone trabeculae lined with osteoblasts and abundant connective tissue rich in neoformed capillaries (Fig. 2A). A progressive bone formation was recognized from the 2nd week on, in parallel to decreasing amounts of connective tissue and coagulum remnants (Fig. 2B), culminating with thick bone trabeculae filling most of the alveolar socket by the 3rd week (Fig. 2C). A delay in the osteogenic process was clearly suggested in the alveolus of stressed rats, which presented an apparently smaller bone volume density and larger areas occupied by persisting coagulum remnants (Fig. 2D).

Histometric data confirmed the incremental bone formation in parallel to decreased volume densities of coagulum and connective tissue 7 to 21 days after tooth extraction. Prolonged intermittent immobilization caused a 40% mean decrease in bone volume fraction throughout the experimental period, in addition to significantly higher volume densities of coagulum remnants and a tendency towards increased volumes of connective tissue (Fig. 3).

Discussion

The sustained stress condition brought about by immobilization was attested by significantly elevated plasma corticosterone concentrations. Moreover, not only persistently high corticosterone levels but also a progressive increase from the 7th to the 14th day after tooth extraction showed that the animals did not habituate to repeated presentation of the stressor but rather showed an increased response, indicative of sensitization of the hypothalamus-pituitary-adrenal axis (Briski and Sylvester, 1987; Pitman et al., 1990).

A previous study from our laboratory revealed that a 6 hours/day immobilization protocol promotes likewise a persistent stress with minor disorders in food intake and body weight gain. Immobilized male rats presented a small reduction (15.0±1.4 %) in food intake in addition to a progressive and proportional decrease (up to 14-15%) in body weight gain from the 10th day on, while pair-fed animals presented only a minor decrease in body weight gain (Almeida et al., 1998a). This effect of prolonged intermittent immobilization was discussed in

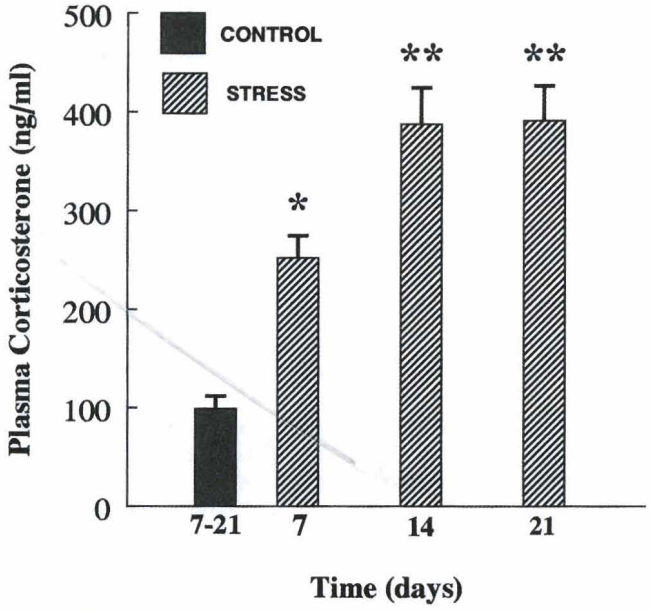

Fig. 1. Plasma corticosterone concentrations in control and stressed rats 7 to 21 days following tooth extraction (Mean ± SEM). As the mean values for plasma corticosterone observed in the distinct control groups did not differ statistically, all control rats were considered as a single control group. Statistically different from the control group (Kruskal-Wallis test): * α = 0.05; ** α = 0.01.

terms of the metabolic activity of high corticosterone secretion and/or the thermogenic effect of noradrenaline. In the present experiment, besides being submitted to a shorter daily immobilization, stressed animals exhibited a significant delay in reparative bone formation from the 7th day after tooth extraction on. Thus, it seems conceivable to assume that any possible change in the nutritional state of immobilized rats would have only a minor contribution to disturb the alveolar healing process, if so.

The intra-alveolar bone healing progressed normally in control rats closely resembling literature data, including those from our laboratory (Brentegani et al., 1996; Lamano Carvalho et al, 1997a,b). The rat alveolar healing occurs by a mechanism for cancellous bone formation in the absence of a precursor cartilagenous tissue, being generally considered a reiterated process of tissue embryogenesis (Jahangiri et al., 1998). The

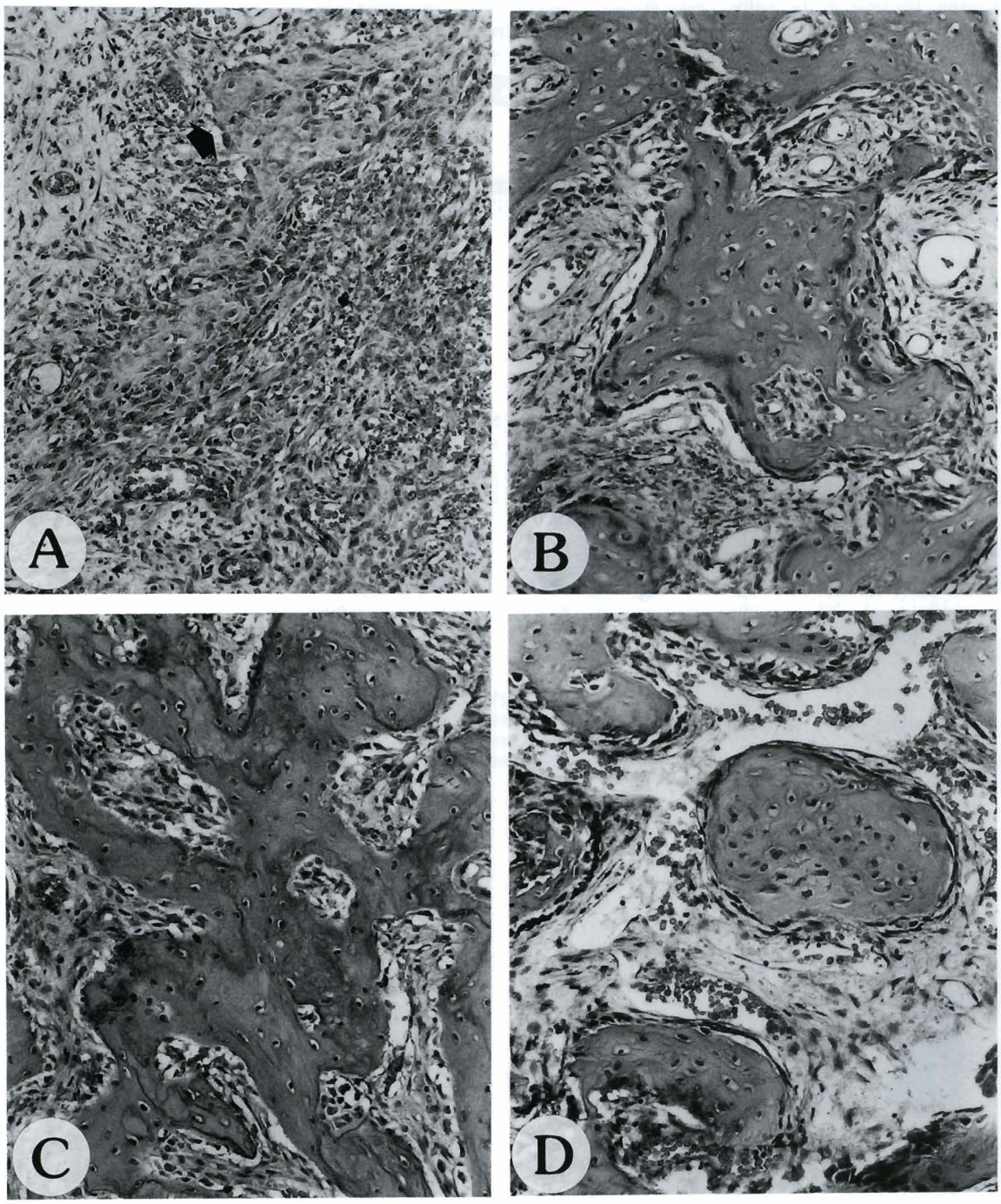

Fig. 2. Rat alveolar socket. **A.** Control rat 7 days after tooth extraction, showing immature trabecular bone lined with osteoblasts (arrow) side by side with connective tissue rich in neoformed capillaries; control rats 14 **(B)** and 21 days **(C)** after tooth extraction, exhibiting a progressive increase in new bone formation. **D.** stressed rat 21 days after tooth extraction, showing a smaller volume density of reparative bone. HE, x 56

coagulum filling the alveolar socket immediately after tooth extraction is progressively reabsorbed, as it is invaded by fibroblasts derived from the residual periodontal ligament which actively proliferate, migrate into the blood clot (Lin et al., 1994) and produce collagen type I and III fibers that create a template for subsequent bone formation (Devlin, 2000). This immature connective tissue (granulation tissue), formed from the socket margins towards the center, is gradually replaced by a more mature connective tissue and latter by new bone trabeculae formed in the same centripetal direction (Jahangiri et al., 1998; Devlin, 2000). There is evidence that both fibroblasts from the periodontal ligament (Lin et al., 1994) and osteoprogenitor cells from the surrounding bone (Devlin, 2000) may be important for osteoblastic differentiation and subsequent new bone production during socket healing. Histometric results in the present experiment attested a significant delay in reparative bone increment in stressed animals, somewhat related to impairment of coagulum remission and/or organization.

Each stage of bone healing, as is also the case for physiological bone growth, is enabled and controlled by a number of priming, proliferating, migrating and differentiating local agents (Bilezikian et al., 1996c for references) and hormones (Bilezikian et al., 1996a,b for references). Among the hormones, glucocorticoids can either stimulate or inhibit bone formation depending on the experimental model and plasma concentration. Low (physiological) concentrations of glucocorticoids

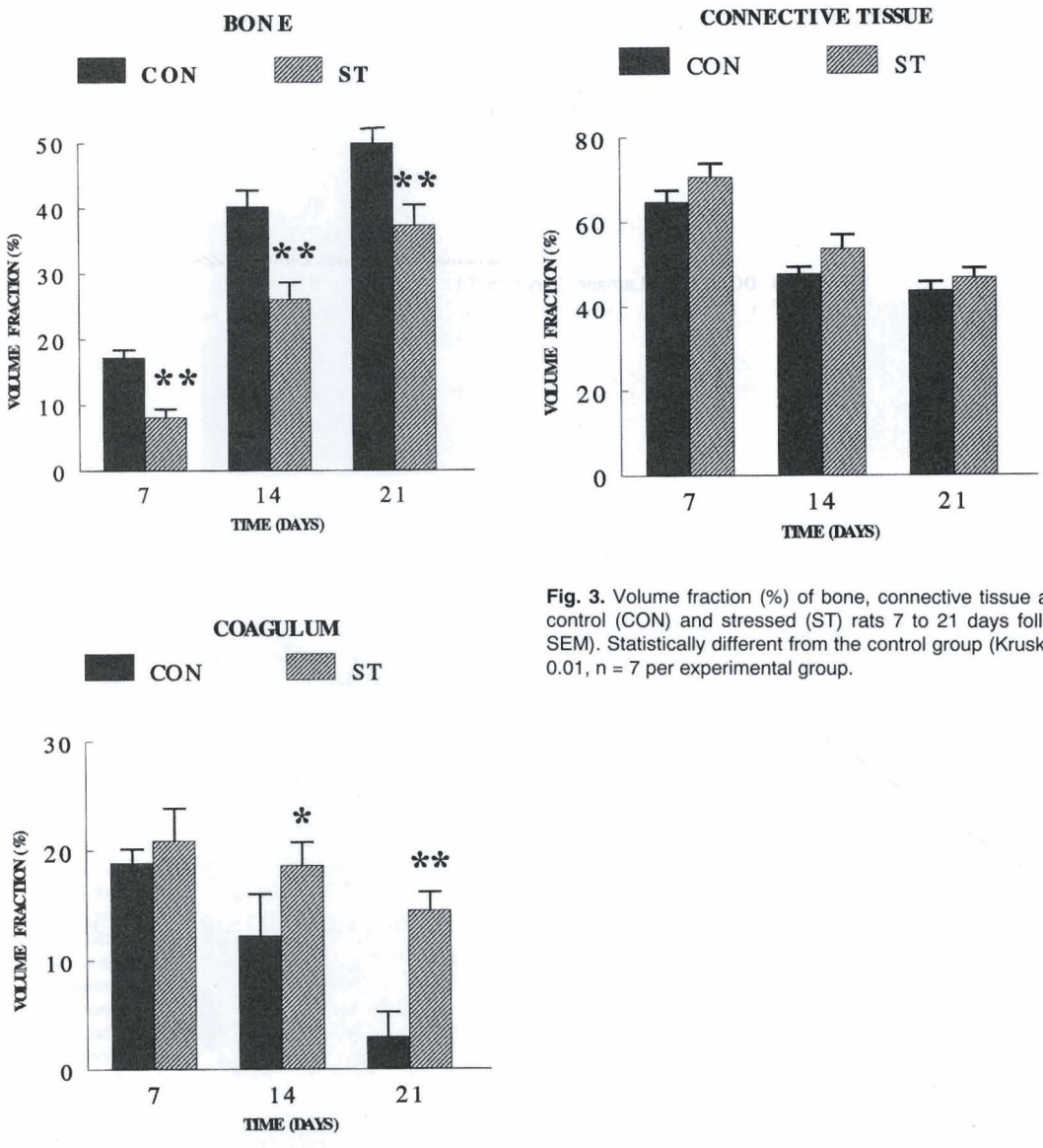

Fig. 3. Volume fraction (%) of bone, connective tissue and coagulum in the alveolus of control (CON) and stressed (ST) rats 7 to 21 days following tooth extraction (Mean ± SEM). Statistically different from the control group (Kruskal-Wallis test): * $\alpha = 0.05$; ** $\alpha = 0.01$, n = 7 per experimental group.

facilitate the action of other hormones, allowing the latter to have optimal activity and being associated with increased osteoblastic differentiation and bone production. Pharmacological doses, however, seem to suppress bone formation by inhibiting osteoprogenitor proliferation and, particularly, by reducing the amount of type I collagen for bone matrix development (Lukert and Kream, 1996, for references). High glucocorticoid levels can have harmful effects from the initial steps of inflammation (vascular permeability and leucocyte migration) up to the subsequent phases of the healing process (coagulum organization, fibroblasts and blood vessels proliferation, collagen deposition) (Saito et al., 1997; Webster et al, 1998).

Thus, although the mechanism(s) by which stress has caused a delay in alveolar bone healing has not been determined, increased corticosterone levels may have contributed. Not surprisingly, preliminary results from our laboratory have shown that benzodiazepine treatment applied to rats concomitantly with immobilization, while moderating plasma corticosterone, seems to promote a partial recovery of intra-alveolar reparative bone density (Bombonato et al., 1998).

It should be pointed out that besides promoting high corticosterone levels, stress can also impair the secretion of a range of hormones (thyroid and growth hormones, prolactin and androgens) (Armario and Jolin, 1989; Almeida et al., 1998b) which are known to regulate bone formation and repair (Bilezikian et al., 1996b for references).

In conclusion, prolonged intermittent immobilization increased plasma corticosterone and hindered rat alveolar bone healing. To our knowledge, this is the first quantitative report confirming the deleterious effect of continued stress on reparative bone formation.

Acknowledgements. The authors thank Dr. C.R. Franci for providing the facilities for hormone measurements. Research supported by FAPESP (96/5745-7) and CNPq (520156/95-1).

References

Almeida S.A., Anselmo-Franci J.A., Rosa e Silva A.A. and Lamano Carvalho T.L. (1998a). Chronic intermittent immobilization of male rats throughout sexual development: a stress protocol. Exp. Physiol. 83, 701-704.

Almeida S.A., Petenusci S.O., Anselmo-Franci J.A., Rosa e Silva A.A. and Lamano Carvalho T.L. (1998b). Decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty. Braz. J. Med. Biol. Res. 31, 1443-1448.

Armario A. and Jolin T. (1989). Influence of intensity and duration of exposure to various stressors on serum TSH and GH levels in adult male rats. Life Sci. 44, 215-221.

Bilezikian J.P., Raiz L.G. and Rodan G.A. (1996a). The hormones of bone. In: Principles of bone biology. Section D. Chap. 23-35. Academic Press. London. pp 305-506.

Bilezikian J.P., Raiz L.G. and Rodan G.A. (1996b). Other systemic hormones that influence bone metabolism. In: Principles of bone biology. Section E. Chap. 36-42. Academic Press. London. pp 507-606.

Bilezikian J.P., Raiz L.G. and Rodan G.A. (1996c). Local Regulators of Bone. In: Principles of bone biology. Academic Press. London. Section F, Chap. 43-53, pp. 607-756.

Bombonato K.F., Lamano Carvalho T.L. and Brentegani L.G. (1998). The influence of a benzodiazepinic in the alveolar healing of stressed rats. J. Dent. Res. 77, 1168. Divisional Abstracts: Brazilian Division (Abstract 207).

Brentegani L.G., Bombonato K.F. and Lamano Carvalho T.L. (1996). Immediate implantation of glass-ionomer cement granules increases osteogenesis during rat alveolar wound healing. J. Nihon Univ. Sch. Dent. 38, 141-145.

Briski K.P. and Sylvester P.W. (1987). Effects of sequential acute stress exposure on stress-induced pituitary luteinizing hormone and prolactin secretion. Life Sci. 41, 1249-1255.

Devlin H. (2000). Early bone healing events following rat molar tooth extraction. Cell Tissues Organs 167, 33-37.

Elenkov I.J., Webster E.L., Torpy D.J. and Chrousos G.P. (1999). Stress, corticotropin-releasing hormone, glucocorticoids, and the immune/inflammatory response: acute and chronic effects. Ann. NY Acad. Sci. 22, 1-11.

Jahangiri L., Devlin H., Ting K. and Nishimura I. (1998). Current perspectives in residual ridge remodeling and its clinical implications: a review. J. Prosthet. Dent. 80, 224-237.

Lamano Carvalho T.L., Bombonato K.F. and Brentegani L.G. (1997a). Histometric analysis of rat alveolar wound healing. Braz. Dent. J. 8, 9-12.

Lamano Carvalho T.L., Teófilo J.M., Araújo C.A.C.A. and Brentegani L.G. (1997b). Chronology of alveolar healing following immediate implantation of *Ricinus communis* polyurethane resin: histometric analysis in rats. J. Biomed. Mater. Res. 37, 449-452.

Lin W-L., McCulloch C.A.G. and Cho M-I. (1994). Differentiation of periodontal ligament fibroblasts into osteoblasts during socket healing after tooth extraction in the rat. Anat. Rec. 240, 492-506.

Lukert B.P. and Kream B.E. (1996). Clinical and basic aspects of glucocorticoid action in bone. In: Principles of bone biology. Bilezikian J.P., Raisz L.G. and Rodan G.A. (eds). Academic Press. London. pp. 533-548.

Munck A. and Guyre P.M. (1989). Glucocorticoid physiology and homeostasis in relation to anti-inflammatory actions. In: Anti-inflammatory steroid action - Basic and clinical aspects. Scheinmer R.P., Claman H.N. and Dronsky A.L. (eds). Academic Press. London. pp 31-47.

Pitman D.L., Ottenweller J.E. and Natelson B.H. (1990). Effect of stressor intensity on habituation and sensitization of glucocorticoid response in rats. Behav. Neurosci. 104, 28-36.

Saito T., Tazawa K., Yokoyama Y. and Saito M. (1997). Surgical stress inhibits the growth of fibroblasts through the elevation of plasma catecholamine and cortisol concentrations. Surg. Today 27, 627-631.

Webster E.L., Torpy D.J., Elenkov I.J. and Chrousos G.P. (1998). Corticotropin-releasing hormone and inflammation. Ann. NY Acad. Sci. 840, 21-32.

Accepted January 15, 2001