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THE USE OF BISPHOSPHONATES TO PROMOTE HEALING OF RAT CALVARIAL WOUNDS

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Dentistry
University of Toronto

I wish to dedicate this thesis to my dear wife Nancy for her confidence, encouragement and unconditional support. I could not have accomplished this without you.

The Use of Bisphosphonates to Promote Healing of Rat Calvarial Wounds

Master of Science, 1998

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Abstract

Previous studies have demonstrated an inverse relationship between bone mineralization and bone matrix formation *in vitro*, and further, that reversible inhibition of mineralization with a bisphosphonate, Etidronate (HEBP), will stimulate bone formation *in vitro*. In this investigation, a rat calvarial wound healing model was used to determine whether reversible inhibition of mineralization with high dose HEBP (15 mg/kg) would accelerate wound closure *in vivo*. Bilateral wounds ($5mm^2$) were made in the calvaria of 150 mg male Wistar rats under general anesthesia. Daily subcutaneous injections of HEBP or saline (control) were administered for one week, followed by a one-week recovery period to permit mineralization. Another group received a second generation bisphosphonate, Pamidronate (APD), at 1 mg/kg, a dose that inhibits resorption but not mineralization. In one group of HEBP treated rats, APD was administered in the second week to inhibit potential resorption of newly formed bone. In some animals, HEBP was administered throughout the two-week period. Histomorphometric assessment of wound closure was carried out on undemineralized serial sections stained with toluidine blue and

the von Kossa stain to visualize osteoid matrix and mineralized bone respectively. At one week, there was a two-fold increase in closure in HEBP treated rats as compared to saline control or APD. The greatest closure was observed in rats treated with HEBP in the first week followed by APD in the second week. While elevated levels of osteoid and osteopontin as well as lowered levels of bone sialoprotein were observed after one week of HEBP treatment, they returned to normal after cessation of HEBP treatment. These data show that wound closure can be accelerated in rats when HEBP is used for a short period of time in a dose high enough to inhibit mineralization reversibly.

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General Introduction and Statement of the Problem

I.1. Introduction

Osseointegration, the growth of bone into an implant surface leading to its stable anchorage, has revolutionized the approach taken by dental professionals in the treatment of edentulism. Titanium tooth root analogues known as dental implants have proven to be successful in the management of these patients (Adell et al. 1990) and innovative approaches in perfecting this type of treatment are being developed continuously. These improvements are aimed at function and esthetics of the restored dentition as well as increasing patient satisfaction and long term success. Despite impressive gains made in this field in the past two decades, the one factor that has not changed is the long waiting period required for the bone to heal around the implants allowing for osseointegration to occur. Following surgical implantation, dental implants are typically submerged subperiosteally, being left dormant for an average period of six months. They must then be re-exposed prior to their final restoration with prosthetic teeth. In order to reduce the waiting period between the initial placement of the implants and the restorative phase, it has been proposed that the implants be left exposed at the time of the initial surgery, thereby eliminating the six month re-entry surgery and its associated healing period (Buser et al. 1990). To a certain limited extent this does expedite the process, but the limiting factor is still the six month delay required for osseointegration to occur before the implants can be restored and loaded.

Clearly, it would be greatly advantageous if this bone healing, or osseointegration period could be shortened in a reliable and predictable manner. If this acceleration of

bone healing were to occur, it could be adapted to and benefit other clinical applications such as bone graft healing and fracture healing in the fields of periodontal, maxillofacial and orthopedic surgery. However, in order to pursue these goals, a clearer understanding of bone cell biology is essential.

I.2. Bone

I.2.a. General background

Bone is a mineralized connective tissue responsible for providing structural support and protection for soft tissue organs in the body and serving as a reservoir for calcium and phosphorous ions. It is a highly dynamic tissue, constantly undergoing remodeling in order to maintain its biomechanical properties and structural integrity. A number of cells are involved in this ongoing remodeling process, including osteoclasts, osteoblasts, osteocytes and bone lining cells.

The osteoblast is the cell responsible for the formation of bone. Osteoblasts first produce the organic bone matrix (osteoid) and subsequently regulate its mineralization (Robey 1985). In addition to synthesizing Type I collagen, the osteoblast produces non-collagenous matrix proteins, osteocalcin, osteonectin as well as numerous growth factors (Puzas 1990). The osteoblast can become entrapped in newly synthesized bone and become an osteocyte. Such cells have numerous processes that extend to form a network in the bone allowing for contact between osteocytes as well as contact with the

bone surface. Alternatively, once an osteoblast has ceased its bone forming activity, it may also become a bone lining cell. Such long flat cells are found covering a large proportion of "quiescent" bone surfaces, but their exact functions are not clear.

The osteoclast is a large multinucleated cell responsible for the resorption of bone. It is attached to the surface of bone where it forms a sealed environment. Active osteoclasts have a ruffled border facing this sealed space, into which is secreted hydrogen ions that lower the pH and promote demineralization of the underlying bone (Mundy 1990). In addition to this, the cell releases proteases that digest the organic components of the bone such as collagen thereby creating a resorption pit known as a Howship's lacunae. Being a multinucleated cell, the osteoclast is derived from the fusion of mononuclear precursor cells of hematopoietic origin (Roodman et al. 1985).

I.2.b. The bone matrix

The extra cellular organic matrix of bone is compromised mainly of Type I collagen (Puzas 1990). In addition to collagen, the matrix contains non-collagenous proteins such as osteocalcin, osteonectin, bone sialoprotein, as well as numerous other proteoglycans and glycoproteins (Termine 1988). There is also a mineral or inorganic component to bone composed of calcium and phosphorous mainly in the form of hydroxyapatite. This mineral phase offers strength to the bone as well as being a

reservoir of various mineral ions such as calcium, magnesium and phosphorus that play a role in mineral homeostasis.

I.2.c. Bone formation

The skeleton is composed of both cortical bone and cancellous bone. Cortical bone is the dense outer layer of both long and flat bones. Cancellous bone is located inside the cortical bone shell and has a more open structure than cortical bone, consisting of trabeculae separated by marrow spaces. Depending on the bone's mechanism of formation, it is referred to as either having been formed via intramembranous or endochondral ossification (Baron 1990). The formation of endochondral bone involves the initial formation of a cartilage model, which is subsequently replaced by bone. On the other hand, intramembranous ossification does not require the intermediate step of cartilage formation (Jee 1983), and so mesenchymal cells differentiate directly into osteoblasts that produce bone.

I.2.d. Bone modeling and remodeling

Bone is a very dynamic tissue in that it is capable of growth, modeling and remodeling as well as healing following wounding. If osteoclastic resorption of bone and subsequent osteoblastic bone formation occur at different sites, the end result is a change in the shape of the bone. This is known as bone modeling and allows for bone growth as well as redistribution of bone mass in order to accommodate changes in load to the bone (Ellis 1981). If resorption resulting from osteoclastic activity is balanced with an equivalent amount of new bone, renewal of bone occurs, known as bone

remodeling. This allows for proper biomechanical maintenance of bone as well as assuring a metabolically and biologically sound bone. In its simplest form, remodeling can be described as a sequence of three phases: 1) activation phase, 2) reversal phase and 3) formation phase (Frost 1969). The first phase is the activation of osteoclast progenitor cells to proliferate into osteoclasts that resorb an area of bone. This is followed by the reversal phase wherein osteoclasts may breakdown thereby giving rise to a variety of mononuclear cells, the function of which is not clear. Subsequently, there is recruitment of osteoblasts that lay down osteoid in the resorption pits during the formation phase. In order to maintain skeletal mass, the remodeling sequence must be tightly controlled, in that an equivalent level of bone formation must follow bone resorption. The homeostatic relationship between bone resorption and bone formation is mediated by mechanisms known as "coupling" (Frost 1969). Coupling and other aspects of bone modeling and remodeling may be regulated by a variety of systems and local factors briefly outlined below.

I.2.e. Regulation of bone metabolism

There are three main groups of factors that may influence bone cell metabolism (Smith 1984). For example, calcium and phosphate regulating hormones such as parathyroid hormone, mineralocorticoids, vitamin D and calcitonin affect systemic regulation. Importantly, local mechanisms relying on the action of cytokines and growth factors (e.g. IL-1, TGF b, TNF, PDGF) which regulate the differentiation and proliferation of osteoclasts and osteoblasts (Canalis 1990). Finally, mechanical forces also play a role in the continuous balance between formation and resorption (Frost 1987). It is beyond

the scope of this thesis to delve into the details of endocrine, autocrine, paracrine and force mediated regulation of bone cell and tissue metabolism. Suffice it to say the concerted action of these three groups of factors governs bone metabolism.

I.2.f. *In vitro* studies

Given the complexity of bone tissue formation and metabolism, a variety of *in vitro* model systems have been developed in order to further study these events. In our laboratory, the chick periosteal osteogenesis (CPO) model has been exploited to study a variety of issues pertaining to bone cell differentiation, bone matrix formation and mineralization (Tenenbaum 1981; Tenenbaum et al. 1989). One of the more intriguing findings obtained with the CPO relates to mineralization of bone. Using the CPO, it was demonstrated that the addition of the organic phosphate β–glycerophosphate (GP) reliably induced mineralization of osteoid that had been formed *in vitro* (Tenenbaum 1981) which was virtually indistinguishable from mineralization that occurred *in vivo*. In addition to its effect on mineralization, cultures exposed to GP appeared to have reduced osteoblastic production of alkaline phosphatase as compared to control osteoblasts (Tenenbaum et al. 1989). Notably, GP treated osteoblasts also produced less bone matrix than did controls. It therefore appeared that when osteoblasts were exposed to GP and subsequently induced to mineralize the bone matrix, they lost their bone matrix producing ability. This suggested that an inverse relationship exists between osteoblast bone matrix production and mineralization in that bone matrix formation and mineralization did not take place concurrently.

This series of *in vitro* observations lead to the following question: Would intentional but reversible inhibition of mineralization lead to increased bone formation *in vitro*, and could this ultimately be exploited *in vivo*? In examining this issue, it became clear that a pharmaceutical agent was needed that might be administered for a short period of time to inhibit mineralization and that when administration stopped, mineralization would then occur. It appeared that a drug from the bisphosphonate family would be the best suited for this, as will be discussed below.

I.3. Bisphosphonates

I.3.a. General background

Bisphosphonates are synthetic analogues of pyrophosphates, which, like pyrophosphates, have a high affinity for hydroxyapatite in bone. They are capable of inhibiting formation and aggregation of calcium phosphate crystals (Fleisch 1968) as well as inhibiting dissolution of hydroxyapatite (Fleisch 1969). As pharmaceutical agents, they have been established in the treatment of Paget's disease of the bone (Hosking 1990; Russell et al. 1974), hypercalcemia of malignancy (Mosekilde et al. 1991), and osteoporosis (Watts 1994; Watts et al. 1990). Bisphosphonates have a phosphorous-carbon-phosphorous structure where the central carbon atom replaces the oxygen atom in the phosphorous-oxygen-phosphorous structure of pyrophosphates, preventing their hydrolysis by pyrophosphatases. Substitution of the different side chains off the central carbon produces unique bisphosphonates with differing

properties. The first generation bisphosphonate 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) has short alkyl side chains, while second generation bisphosphonates such as (APD) are characterized by side chains with amino terminal groups. As new drugs are developed, they often exhibit bone resorption inhibiting properties of 10-100 times the previous generation (Sietsema et al. 1989), making them in effect more potent, thereby allowing lower doses to be used. In contrast, the newer generations of bisphosphonates vary little in their mineralization inhibitory activity (Fleisch 1997), but given that they can be used in much lower doses than earlier generation bisphosphonates, their inhibitory effects on mineralization are thus minimized. As previously mentioned, bisphosphonates have two distinct effects on bone formation and turnover: at a low dose, they inhibit bone resorption, and at a high dose, the bisphosphonates will also cause impairment of bone mineralization. With HEBP, the dose level required to inhibit osteoclast mediated bone resorption *in vivo* is nearly equivalent to the dosage that also inhibits bone mineralization (Fleisch 1993; Heany et al. 1976). This narrow therapeutic range has limited the use of HEBP in various clinical applications, as a low dose of 5 mg/kg/day aimed at reducing bone turnover may be associated with focal defects in bone mineralization (Weinstein 1982; Boyce et al. 1984). By increasing the dose to only 10 mg/kg/day, complete inhibition of mineralization may occur (Hosking 1990). In fact, many original studies reported that this impaired mineralization resulting in accumulation of osteoid similar to osteomalacia, was a common side effect with the administration of HEBP in high doses (10-20 mg/kg/day) (Boonekamp et al. 1986; DeVreis et al. 1974; Russell et al. 1974; Smith et al. 1973). In order to circumvent this "undesired" side effect, HEBP is

prescribed in a cyclical regimen instead of being administered in a continuous fashion (Watts et al. 1990). Moreover, as this inhibition of mineralization was considered a deleterious side effect, newer bisphosphonates such as APD were developed which produce little or no inhibition of mineralization at doses which still inhibit resorption (Fleisch 1991), as alluded to above. Inhibition of resorption could therefore be achieved at a dosage greatly reduced from that required to effectively block mineralization. This explains why these bisphosphonates are considered more potent agents than HEBP, and are therefore often favored for therapeutic purposes.

I.3.b. Pharmacodynamics

Oral bioavailability of bisphosphonates is considered to be very poor. It has been demonstrated with radiolabeled bisphosphonates that bioavailability following oral administration in animals and humans is as low as 1 to 5% of the dosage (Recker et al. 1973; Lin et al. 1991). The absorbed drug is cleared from the blood very rapidly by adsorption onto bone and excretion in the urine in its unchanged form (Michael et al. 1972). Following intravenous administration of bisphosphonates in animals and humans, the drug disappeared rapidly from plasma with a half-life of only 1-3 hours (Fitton et al. 1991; Dittert 1977). Its distribution in bone varies since proportionally more will be deposited at sites of bone formation (Fogelman et al. 1981). Parenthetically, this targeting effect could have great advantages in sparing non-affected sites of the skeleton if the drug is used to accelerate healing in state of injury. It's been reported that HEBP labels five times more osteoblast surface than does Alendronate, a third generation bisphosphonate, in addition to labeling approximately

equal fractions of osteoclast and osteoblast surface (Masarachia et al. 1996). On the other hand, Alendronate labeled eight-fold more osteoclast than osteoblast surface. Bisphosphonates adhered to hard tissues are very gradually eliminated, as its been reported that their half life varies from 200 days in laboratory animals to 10 years in humans (Gertz et al. 1993; Lin et al. 1991).

I.3.c. Effects on bone

I.3.c.i Effects on bone resorption:

It was originally thought that bisphosphonates inhibited bone resorption through physicochemical interactions with hydroxyapatite (Fleisch 1969). It is now accepted that the bisphosphonates have a variety of effects on cells thought to be involved in the resorption of bone such as osteoclasts (Flanagan et al. 1989) or their precursors (Boonekamp et al. 1987). It has also been suggested that part of the inhibiting action on the osteoclasts is mediated indirectly through action on the osteoblasts (Sahni et al. 1993). A variety of effects of the bisphosphonates on the cellular biochemistry of the osteoclast *in vitro* have been described (Fleisch 1991), but no single explanation fully describes the anti-resorptive mechanism of action of the bisphosphonates *in vivo*.

I.3.c.ii. Effects on bone mineralization:

One of the unique chemical properties of bisphosphonates relates to their ability to bind onto calcium salts, in particular hydroxyapatite. This physico-chemical adsorption onto hydroxyapatite results in blockage of the crystal growth (Fleisch 1969; Francis 1969), thereby inhibiting mineralization, although not completely. In addition to *in vitro*

inhibition of apatite crystal growth, bisphosphonates effectively inhibit calcification *in vivo* as they prevent experimentally induced ectopic ossification and calcification of soft tissues (Fleisch et al. 1970). As noted above, numerous studies have described the inhibition of mineralization from therapeutic use of HEBP (Boonekamp et al. 1986; DeVreis et al. 1974; Russell et al. 1974; Smith et al.1973), and how it is considered a deleterious side effect. This inhibition of mineralization has been demonstrated to be reversible both *in vivo* (King et al. 1971) and *in vitro* (Tenenbaum et al. 1992). Historically it has been accepted that this inherent ability for bisphosphonates to block mineralization is purely a physico-chemical interaction with bone. It has, however, been suggested that their mode of action could be through direct interactions with bone producing cells, the osteoblasts (Francis et al. 1983). In order to investigate the bisphosphonates' mechanisms of action on mineralization, and to address the question elucidated to above, this laboratory has conducted a variety of *in vitro* studies using the chick periosteal osteogenesis (CPO) model as described below.

I.3.d. Effects of HEBP on osteogenesis *in vitro* using the CPO

It is clear then that one of the effects of bisphosphonates and in particular HEBP, on mineralization is that of inhibition which may be reversible (Tenenbaum et al. 1992; King et al. 1971). As noted above, it was hypothesized that a drug with such actions might be used to effectively stimulate bone formation if used in a pulsatile manner, due to the apparently inverse relationship between bone matrix formation and its mineralization.

This lead to a series of studies in our laboratory that focussed on the following questions:

1) Would HEBP inhibit glycerophosphate-induced mineralization *in vitro*?
2) Was this inhibition reversible, and what was the nature of the mineral formed after cessation of HEBP treatment?
3) Did bisphosphonates used at this dose remain in the matrix or was it found only in the mineral?
4) Could reversible inhibition of GP-induced mineralization lead to increased matrix and bone formation *in vitro*?

With the use of the CPO, it was confirmed that HEBP reversibly inhibited mineralization of bone formed *in vitro* (Tenenbaum et al. 1992). Not only was this phenomenon reversible, but when cultures were allowed to mineralize once the HEBP was removed, the density of the mineral formed was actually greater than the mineral density in control cultures (Torontali et al. 1994). The HEBP was also compared in this *in vitro* system to a second generation bisphosphonate, APD, used at a low dose, which has minimal effects on mineralization. When the cultures were prevented from mineralizing in the presence of HEBP, they produced greater amounts of bone matrix than did the cultures exposed to APD, which were allowed to mineralize (Goziotis et al. 1995). APD treated cultures did eventually produce the same amount of bone matrix, but at a greatly reduced rate. These data suggested that temporary inhibition of bone mineralization could be exploited *in vivo* in order to accelerate production of bone matrix and subsequently mineralized bone formation following removal of the HEBP.

To test this hypothesis, a rat calvarial wound-healing model was used in a pilot study. Using this model, it was shown with the use of radiographic assessment that $5mm^2$ calvarial bone wounds appeared to close at least 50% more rapidly in rats that were exposed to HEBP as compared to control (Tenenbaum et al. 1991). The experimental rats were exposed to a high pulse dose of HEBP (15 mg/kg) for a one week period and then followed for a period of up to 30 days. Calvarial wounds in control rats did eventually close, but at a reduced rate.

I.4. Statement of the problem and aim of the study

I.4.a. Hypothesis

Bone formation, and hence, bone wound closure in calvaria will be accelerated about two-fold in rats treated with high pulsatile–dose HEBP and partial resorption of this newly formed bone will be prevented by subsequent administration of a second generation bisphosphonate, APD, at a lower dose.

I.4.b. Rationale

In the above noted *in vivo* study, surgically created defects in the calvaria of rats appeared to close at least 50% more quickly in animals treated with high pulse dose HEBP as compared to control (Tenenbaum et al. 1991), but this was only assessed radiographically. In a more recent investigation, alveolar bone volume was increased

greater than two-fold in rats treated with high pulse dose of HEBP, as used in the calvarial wound study (Lekic et al. 1997). However, when animals were allowed to recover from the HEBP treatment to allow for mineralization of the newly formed bone matrix, it appeared that approximately 20-25% of the newly formed bone was lost. Despite this partial loss of newly formed bone, the drug treated animals still demonstrated considerably greater amounts of alveolar bone volume than control animals (Lekic et al. 1997). It must be recognized that, the data pertaining to matrix synthesis notwithstanding, the increase in bone volume observed following high pulse-dose treatment of HEBP could be related to inhibition of osteoclast activity. Nonetheless, the effects on osteoblast synthesis of matrix cannot be dismissed. However, it is noteworthy that regardless of the underlying mechanism, clear increases in bone volume can be detected which may make this finding clinically relevant. Thus, further investigation of this question is proposed below.

I.4.c. Objectives

I.4.c.i. Objective 1

To confirm the preliminary findings concerning acceleration of bone wound closure in animals treated with HEBP using more rigorous histomorphometric methods, and to characterize HEBP effects on production of bone associated proteins.

I.4.c.ii. Objective 2

To determine if administering APD over the HEBP recovery can prevent the partial loss of HEBP-induced bone volume increases (osteoid) seen in animals treated for one week with HEBP.

I.4.c.iii. Objective 3

To determine whether wound closure will be accelerated following initial treatment with a second generation bisphosphonate, APD, at a dose which will inhibit osteoclastic activity but not mineralization.

I.4.c.iv. Objective 4

To determine *in vivo* if an actual increase in bone matrix production occurs in HEBP treated animals thereby suggesting that accelerated wound closure in these animals is not strictly due to inhibition of osteoclastic activity.

Chapter II

Acceleration of Rat Calvarial Wound Closure with Bisphosphonates

II. 1. Selection of a model

As bone formation is governed directly by osteoblasts, it seems clear that in cases where new bone formation is required (e.g. fracture, wound repair), it would be advantageous to stimulate osteoblast like cells to produce bone. The above noted findings suggested that it might be possible to administer a drug such as HEBP *in vivo* in a high enough pulse dose ostensibly to inhibit mineralization temporarily, thereby enhancing bone matrix growth and ultimately mineralized bone formation when the treatment is stopped.

In order to evaluate the acceleration of bone wound healing, an animal model was required, and a previously described rat calvarial wounding model was chosen (Tenenbaum et al. 1991; Turnbull et al. 1974). It is has been suggested that the bone wound should be large enough to avoid spontaneous healing. In the absence of adjunctive treatment, such wounds simply fill with fibrous connective tissue, and are therefore referred to as critical size wounds or defects. Such a model is typically used for evaluation of locally delivered biomaterials that promote bone regeneration. From a perspective of osseointegration, a critical size dimension for the type of experiment proposed is not appropriate, as the goal here is to replicate a situation which does heal naturally, albeit at a very slow rate, and to attempt to accelerate it. Such a situation would be representative of a surgically placed dental implant in that the implant eventually does osseointegrate, and to reiterate, the goal would be to expedite this integration. As well, from a biological perspective, healing of a critical size wound

likely differs from that of a spontaneously healing smaller defect. In order to stimulate healing in the former situation, the adjunctive experimental treatment would need to be osseoinductive and stimulate proliferation and differentiation of progenitor cells into bone producing cells. In the latter circumstance, stimulation of bone cell activity, as well as differentiation (Lekic et al. 1997) is required and may occur with HEBP treatment. Clearly it would be of interest to study HEBP effects on both a critical size defect and a smaller defect given their differing biological and physiological requirements. However, given my interest in osseointegration where healing does occur spontaneously, and since pilot data where already available in a non-critical size defect study where 5 mm^2 calvarial wounds did eventually close after more than forty days of healing (Tenenbaum et al. 1991), I chose to focus on HEBP effects on healing in a non-critical size defect model. Moreover, as alveolar bone arises due to intramembranous ossification, it is probable that healing occurring in calvarial bone (also intramembranous) should be more comparable to healing expected in the mandible or maxilla.

II. 2. Materials and Methods

II. 2.a. Animal Model

Male Wistar rats, weighing from 130 to 150 mg, (Charles River) were used. During the course of the experiment, the rats were housed in pairs and maintained under a 12 hour light / dark illumination cycle. The animals were fed a standard laboratory chow and

water *ad libitum*. All animals were treated in compliance with regulations from the University of Toronto Comparative Medicine Department, and the University of Toronto animal experimentation ethics committee had approved the research proposal.

Under general anesthesia (Halothane and Nitrous oxide), the hair on the animals' crania were shaved, and the skin disinfected with alcohol and povidone iodine. An incision, approximately 25mm in length was made and a skin flap reflected, exposing the underlying calvarium. This allowed a hole to be drilled in each parietal bone using a burr in a dental handpiece under saline irrigation. To smoothen the margins of the hole, a hand instrument (curette) was used, removing any bone spicules. This produced "through and through" or trephine-like defects, but the underlying dura mater was left undisturbed. Following thorough saline irrigation, the skin flaps were reapposed and sutured with 4-0 Vicryl sutures. On a prophylactic basis, animals were administered Buprenorphine (0.2 mg/kg) as a post-operative analgesic. Animals were monitored closely and kept warm under a heating lamp for one hour following the surgery.

II. 2.b. Drug administration

Starting on the day of the surgery, the animals were administered, by subcutaneous injection, either Etidronate (15 mg/kg), Pamidronate (1 mg/kg) or the vehicle (saline) according to the following schedule:

Table II.1. Distribution of experimental and control groups for calvarial wounding

GROUP	WEEK ONE	WEEK TWO
1	Saline	None (sacrificed day 7)
2	HEBP	None (sacrificed day 7)
3	APD	None (sacrificed day 7)
4	Saline	Saline
5	HEBP	Saline
6	APD	Saline
7	HEBP	HEBP
8	HEBP	APD
9	Saline	APD

These doses had been established from both pilot studies as well as previously published information (Sietsema et al. 1989; Schenk et al. 1986, Tenenbaum et al. 1991)

II. 2.c. Tissue Preparation

The animals were sacrificed by anesthetic overdose (Halothane and Nitrous oxide) at day seven (groups 1, 2 and 3) or day fourteen (groups 4 – 9). In order to label cycling cells, the rats were injected intraperitoneally with ^{3}H-thymidine (1μCi/g) diluted in PBS to 1ml one hour prior to sacrifice. Following sacrifice, the calvariae were resected and one calvarial half was fixed (see below II.2.c.ii) for preparation of undemineralized

sections by embedding in plastic while the other half was fixed (see below II.2.c.i) for preparation of the demineralized sections by embedding in paraffin.

II. 2.c.i. Demineralized Specimens

The right calvarial halves were fixed in periodate-lysine-paraformaldehyde (McLean et al. 1974) at pH 7.4 for 24 hours at 4 °C, demineralized for 24 hours in 0.2N HCl at room temperature and finally washed in PBS for 20 hours. The specimens were then bisected with a sharp scalpel in the center of the wound. This facilitated embedding and tissue orientation so that sections could be cut from the middle of the wound with very little waste in sectioning time. The calvarial specimens were then dehydrated in a series of graded ethanols, cleared in toluene and embedded in paraffin. With the use of a microtome (Reichert-Jung), 5 micron serial sections were made in a coronal plane, every fifth section being used for morphometric or other analyses. A total of three sections per calvarium were floated in a warm water bath (50-55°C) and lifted onto Superfrost Plus slides which were placed on a warm table (40°C) for ten minutes prior to being incubated (55°C) overnight. These slides were used for immunohistochemistry and autoradiography (see below II.2.d. and II.2.e.).

II. 2.c.ii. Undemineralized Specimens

The left calvarial halves were fixed in 10% neutral buffered formalin under vacuum at room temperature. Specimens were then dehydrated in ascending grades of acetone and then infiltrated with an ascending grade of acetone-spurr mixture. The specimens were then embedded in spurr, which was polymerized in an oven at 53°C. Five micron

sections were obtained in a transverse plane with the use of a 2050 Reichert-Jung microtome and tungsten carbide knife. Specimens were sectioned until the total periphery of the wound was observed on the cross-sections, at which point every third section was chosen for histomorphometry. These sections were stained with von Kossa and Toluidine blue counterstain for demonstration of mineralized and non-mineralized bone (or osteoid) respectively, and were used for morphometric analysis of wound closure and measurement of mineralized bone/osteoid ratios within the wound.

II. 2.d. Immunohistochemistry

Immunohistochemistry was performed to identify bone-associated proteins BSP and OPN. These bone-associated proteins were identified in order to assist in further elaborating on the possible mechanism of action of the bisphosphonates in this wound-healing model. Similar protocols were used for both proteins. The sections were deparaffinized in toluene and then dehydrated in a graded series of ethanols. After rinsing in PBS, the slides were incubated with 3% H_2O_2 for 30 minutes in absence of light in order to inactivate endogenous peroxidase activity. This was followed by incubation with casein blocking solution for one hour in order to reduce non-specific background staining. Specimens were then incubated with the primary antibody for 3 hours then with the secondary antibody for 30 minutes. For OPN detection, the primary mouse monoclonal anti-OPN antibody (Hybridoma Bank, Johns Hopkins University, Baltimore, MD) was diluted 1:600 in PBS as was the secondary antibody, biotinylated anti-mouse IgG (Vector, Burlingame, CA). For BSP detection, the primary rabbit polyclonal anti-BSP antibody (kindly provided by Dr. J. Sodek) was diluted

1:100 in PBS while the secondary antibody, biotinylated anti-rabbit IgG (Vector, Burlingame, CA) was diluted 1:600 in PBS. The specimens were than exposed to Strept ABComplex/HRP (Avidin Biotinylated Horseradish Peroxidase Complex, Vectastain kit, Vector, Burlingame, CA) for 30 minutes, followed by exposure to diaminobenzidine (DAB, Vectastain kit, Vector, Burlingame, CA) substrate for 15 minutes (Nakane 1974). Sections were stained with haemotoxylin and eosin (H&E) and permanently mounted with permount and a glass cover slip. The intensity of the staining was then assessed semi-quantitatively under light microscopy (Laborlux K, Leitz) and assigned a score from 0 to 3 depending on the level of staining present. Level of staining at the periphery of the wound was evaluated in relation to the intensity of staining for these proteins in adjacent intact bone. When the level of staining within the wound was similar to the non wounded bone, it was classified as 2. If the staining was greater than the non wounded bone, it was classified as 3 while less staining would be 1. A score of 0 was assigned in the absence of any staining. This method has been validated and described earlier (Lekic et al. 1997).

II. 2.e. Autoradiography

Autoradiography for ^{3}H-thymidine and ^{3}H-proline was performed in order to label cycling cells and newly formed matrix respectively (see chapter III for ^{3}H-proline). Wax-embedded specimens previously mounted on slides were deparaffinized in toluene, and dipped in 100% ethanol and allowed to dry for 30 minutes. In the dark room, the slides were individually dipped in radiographic emulsion (Kodak TO-2, Eastman Kodak Co., Rochester, NY) and placed in light-tight, dry boxes and exposed

for two weeks at 4°C (McCulloch et al. 1989). The slides were then developed (D-19 Kodak, Eastman Kodak Co., Rochester, NY), stained with H&E and permanently mounted with a glass cover slip and permount. Sections were examined under light microscopy (Laborlux K, Leitz). The average number of labeled cells per field was measured and reported as such, and so this cannot be considered as a labeling index. A cell was considered to be labeled if more than five silver grains overlaid its nucleus (McCulloch et al. 1989).

II. 2.f. Histomorphometry

Wound closure and quantity of mineralized tissue within the wound were analyzed in mineralized specimens previously stained with von Kossa/toluidine blue, quantified under light microscopy (Matallux 3, Leitz) with the aid of the bone morphometry program of the Bioquant morphometric system (R&M Biometrics, Nashville). All analyses were done in blinded fashion on coded slides in order to reduce the possibility of bias.

II. 2.f.i. Wound Closure

Since there was some variation in initial wound size, calvarial wound closure was quantified by measuring the size of the residual wound opening and reporting this as a percentage of closure from the original size of the wound, as measured on each specimen. The original wound perimeters could be identified very easily in these sections.

II. 2.f.ii. Mineralization

Effects of the bisphosphonates on mineralization within the healing wound was determined by morphometric quantification of mineralized tissue (von Kossa positive...black) and osteoid tissue (von Kossa negative...blue) within the calvarial wounds. These were reported as percentages (mineralized: osteoid area).

II. 2.g. Statistical analysis

All analyses were done using the SAS statistical software package. To assess the effect of the recovery week (i.e. on mineralization of the osteoid) and the effects of the one week treatments jointly, groups one through six were analyzed as a two way analysis of variance (ANOVA). One factor was the treatment (saline, HEBP or APD) and the other the time of sacrifice (1 week or 2 weeks). Numerical variables for groups 1, 2 and 3 were compared using a one way ANOVA. The same procedure was used to compare these parallel groups following the recovery week, as well as to compare the effect of the second week treatment following an initial week of HEBP. Other comparisons between groups designed to test specific hypotheses (i.e. does APD prevent bone loss in the recovery week) were made using t-tests. Based on sample size calculations and on the basis of previous studies using the periodontal window wound model (Lekic et al. 1997), five animals per experimental group was deemed as being required to detect a 50% difference in wound closure with a probability of 90%.

II. 3. Results

II. 3.a. Wound closure

At one week, the HEBP treated group demonstrated a two-fold increase in closure (21.4% closure) as compared to saline (10.9%) and APD (9.9%) (figure II.1) ($p<0.05$). When the three parallel groups were allowed to recover (i.e. no drug treatment) for an additional week, the HEBP treated group still showed the greatest amount of closure (39.1%) compared to APD (30.9%) and saline (22.4%) (figure II.2) ($p<0.05$). Treatment with HEBP, APD or saline during the second week did not significantly increase the closure of the wound ($p>0.05$), but there appeared to be a trend towards greater closure when APD was administered in the recovery week (figure II.2). The difference in wound closure between HEBP treated rats and saline controls can be appreciated in the histological photographs of each group (figures II.5 & II.6).

II. 3.b. Mineralization

In the groups sacrificed after one week of treatment, significantly greater percentages of osteoid to mineralized tissue were found within the wound in the experimental group that had been treated with HEBP ($p<0.05$). This elevated percentage of osteoid to mineral was not observed in the APD or in the saline group (figures II.3, II.5 & II.6). After 1 week recovery (i.e. week 2; no drug treatment), this elevated osteoid to mineral percentage demonstrated in the first week in the HEBP group returned to a level compatible to the saline/saline control group. If the HEBP was continued over the

second week, the osteoid level remained elevated within the wound. There was no statistically significant difference in the percentage of mineral to osteoid between saline control and the group receiving HEBP treatment followed by APD treatment, even though there appears to be a trend towards less mineralization in the experimental group (figure II.4).

II. 3.c. Immunohistochemistry

Levels of bone associated proteins OPN and BSP were determined by immunohistochemistry. One week following drug exposure, the levels of OPN staining were elevated in the HEBP treated groups as compared to saline and APD, but this was not statistically significant (figure II.7). On the other hand, levels of BSP staining were lower in the HEBP treated rats compared to the APD and saline groups, but this was also statistically non significant. After one week recovery, BSP and OPN staining levels in the group receiving HEBP/saline returned to similar levels as the saline/saline and APD/saline groups (figures II.8).

II. 3.d. ^{3}H-thymidine uptake

In order to label cycling cells, rats were injected with ^{3}H-thymidine one hour prior to sacrifice. Rats treated with either bisphosphonates, HEBP or APD, showed a reduction in the total number of cycling cells per field. Both groups had significantly lower number of labeled cells than saline ($p<0.05$), and even though the number of labeled cells in HEBP-treated tissues appears to be lower than in APD-treated tissues, this difference was non-significant (figure II.9). Following the one week recovery period,

the three groups had significantly lowered labeled cell counts in the second week as compared to their respective first week, but the bisphosphonate groups remained significantly lower than saline ($p<0.05$).

II. 3.e. Calvarial thickness

Treatment of rats with HEBP caused significant increase in calvarial thickness as compared to saline and APD (figure II.10). The increase in thickness observed in HEBP treated rats was four times greater than in saline or APD. When rats were allowed a one week recovery period, 27% of this newly formed osteoid was lost, but the thickness remained significantly greater than the saline and APD groups. The administration of APD in week two following initial treatment with HEBP did not prevent any significant loss of this newly formed osteoid ($p>0.05$).

II. 4. Legends, Figures and Tables

Figure II.1. **Calvarial wound closure following 1 week exposure to the bisphosphonates or saline (groups 1,2,3)**

A two fold increase in wound closure occurred in HEBP treated animals ($p<0.05$) compared to APD and saline groups. There was no statistically significant difference between the APD and saline groups. The bars represent the means of the measurements taken from the five rats per group with the vertical lines representing the SD. The * represents a statistically significant difference from control.

Calvarial wound closure one week results

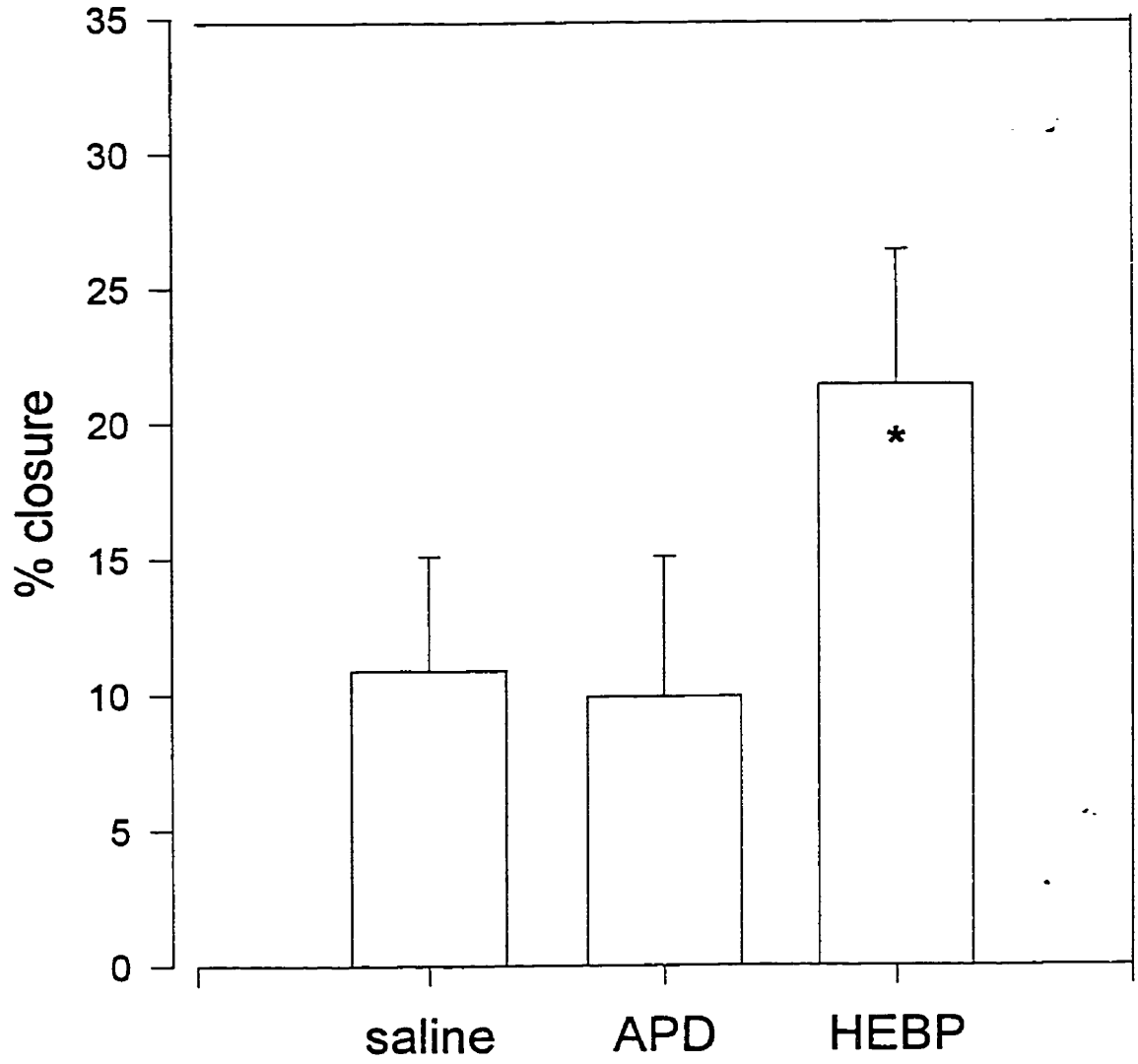


Figure II.2. **Calvarial wound closure following 1 week exposure to the bisphosphonates or saline followed by a 1 week recovery period (groups 4-9)**

Of the groups receiving saline in the recovery week, the group exposed to HEBP in the initial week had the greatest wound closure ($p<0.05$) as compared to APD and saline. In the groups receiving HEBP in the first week, there was no statistically significant increase in wound closure independent of whether HEBP, APD or saline was administered in the second week. There was however a tendency towards greater closure in the HEBP/APD group. Represented are the means of the measurements taken from the five rats per group with the vertical lines representing the SD. The * represents a statistically significant difference from saline control.

Calvarial wound closure two week results

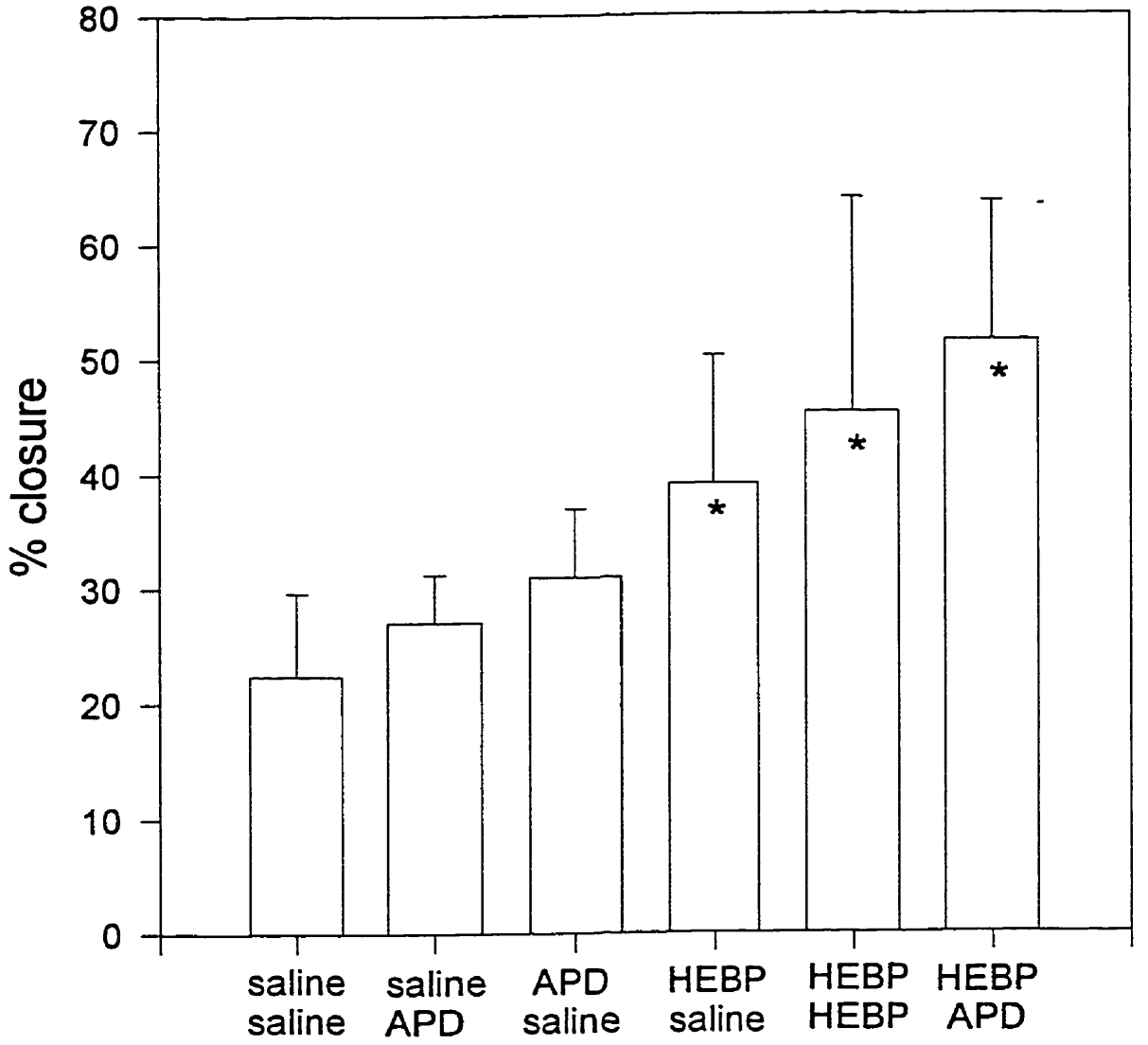


Figure II.3. **Percentage of osteoid to mineralized tissue within the wound one week following exposure to bisphosphonates or saline**

A greater percentage of osteoid to mineral was observed within the healing wound in the HEBP exposed group compared to APD and saline ($p < 0.05$). The APD group had a lower osteoid to mineral percentage than the saline group, but the difference was not significant. Represented are the means of the measurements taken from the five rats per group. The * represents a statistically significant difference from control.

Ratio of osteoid to mineralized tissue within the wound one week

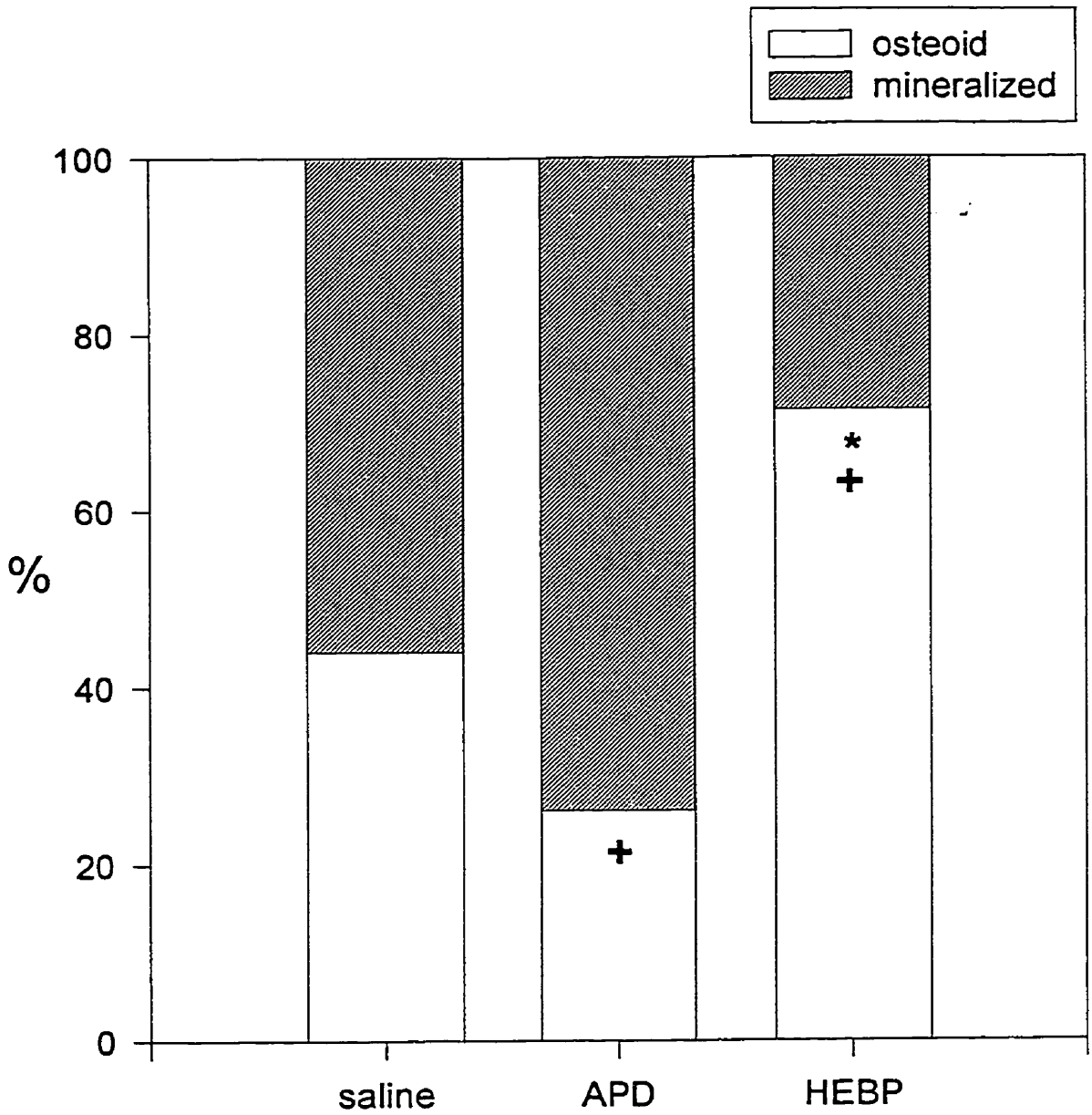
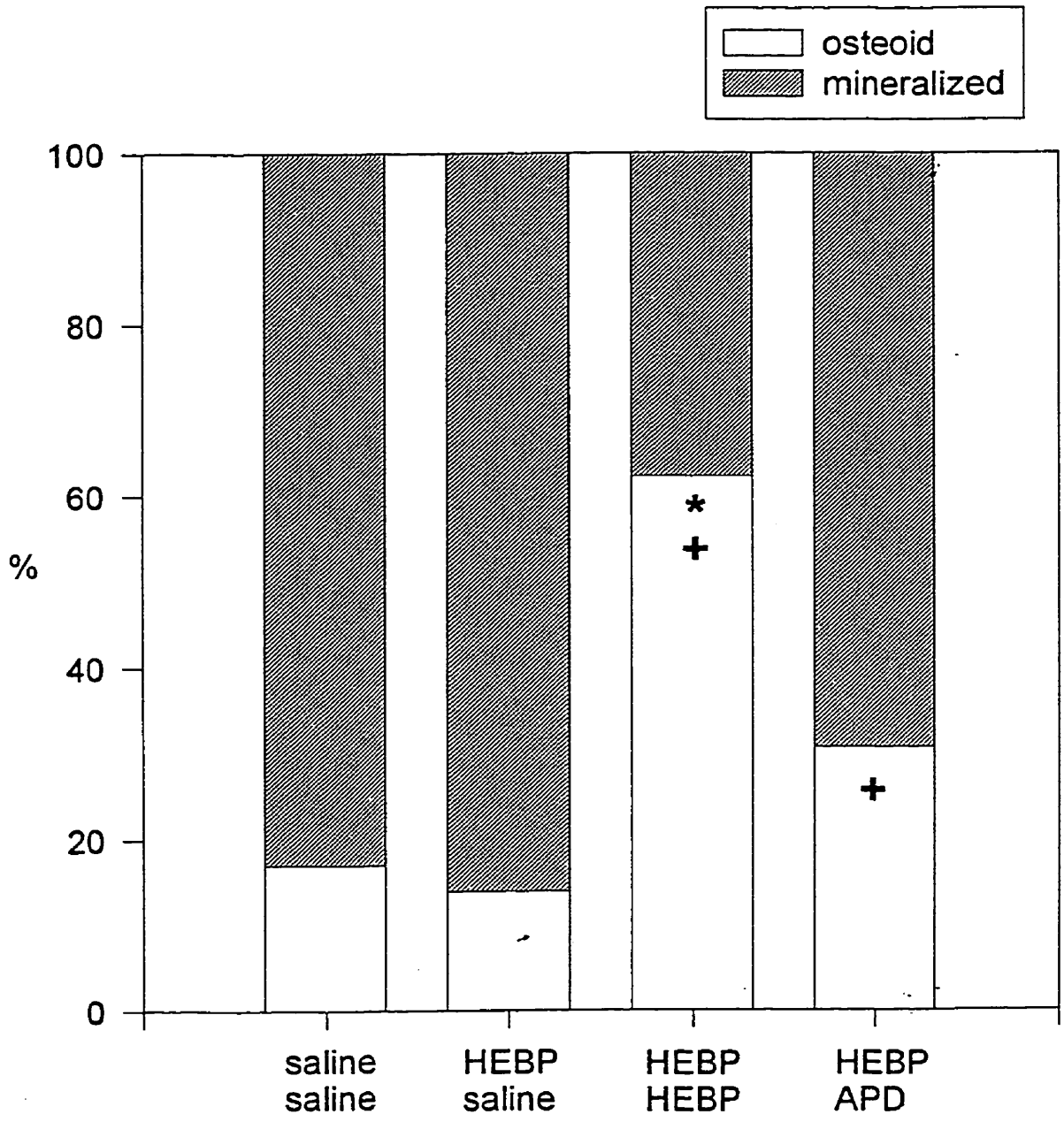


Figure II.4. **Percentage of osteoid to mineralized tissue within the wound after an initial week of drug exposure followed by a recovery week**

When the HEBP treated group was given saline in the recovery week, the elevated levels of osteoid previously noted during week 1 returned to levels similar to saline ($p>0.05$). However, if HEBP was continued for the recovery week, the percentage of osteoid to mineral remained elevated (significantly different from saline, $p<0.05$). If APD was given during the second week, the osteoid level was reduced but it would appear that it did not reach the levels observed in the saline control. There is however no significant difference between these two groups. Represented are the means of the measurements taken from the five rats per group. The * represents a statistically significant difference from control. The + represents a statistically significant difference between groups ($p<0.05$).

Ratio of osteoid to mineralized tissue within the wound two weeks



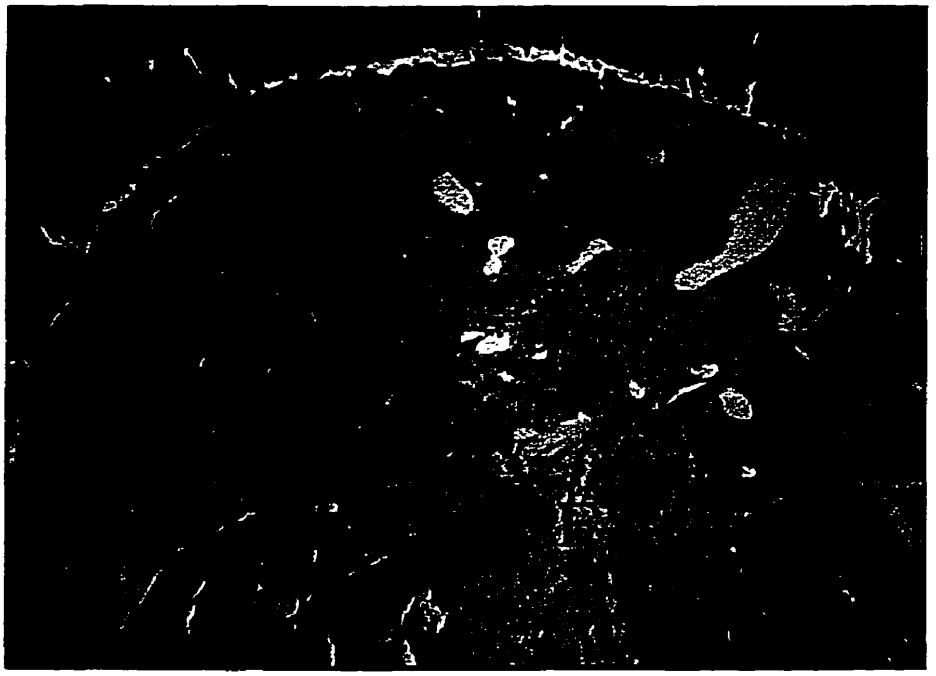


Figure II.5. Calvarial wound closure HEBP-saline
Von Kossa\Toluidine blue staining of a transverse section of the calvarium. The rat was exposed to 1 week of HEBP followed by 1 week of saline. Large amounts of mineralized tissue are present (M) as well as osteoid (O) and fibrous tissue (F). (Magnification 44x)

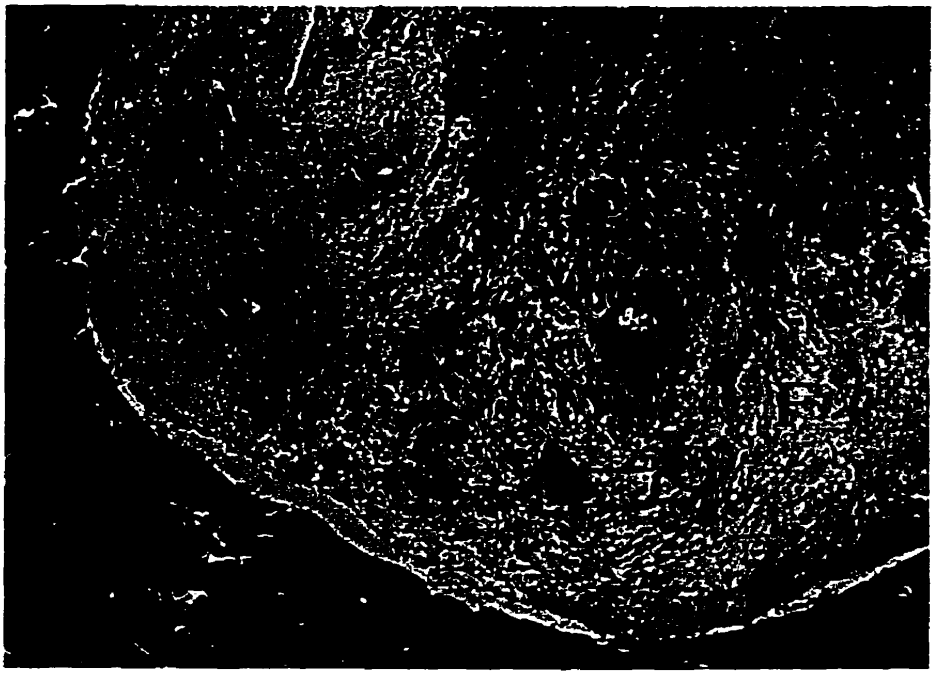
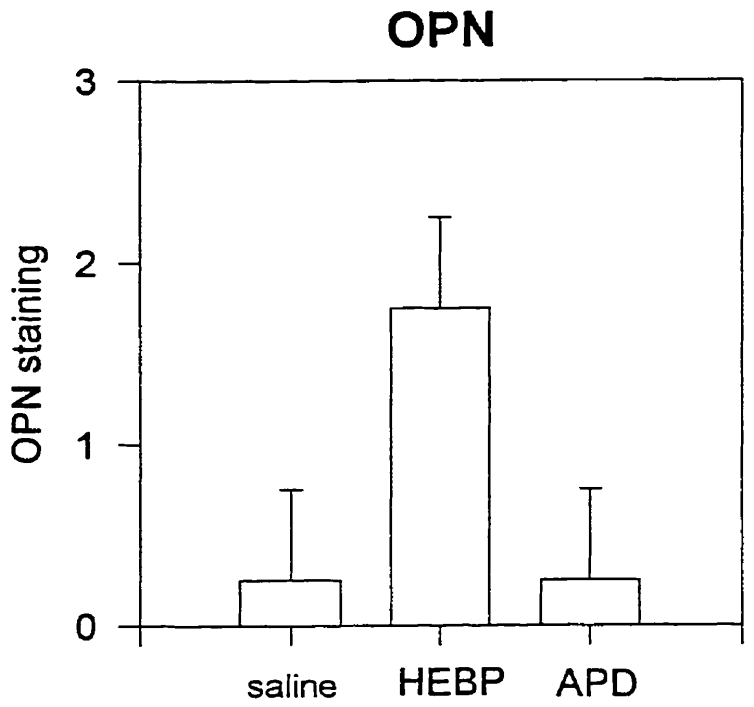


Figure II.6. Calvarial wound closure saline-saline
Von Kossa\Toluidine blue staining of a transverse section of the calvarium. The rat was exposed to 2 weeks of saline. The wound is filled mostly with fibrous tissue(F) but some small areas of mineraliz tissue (M) are present as well as osteoid (O). (Magnification 44x)

Figure II.7. **Levels of bone associated proteins BSP and OPN within the one week healing wound as determined by immunohistochemistry**

Rats sacrificed after one week exposure to HEBP had a tendency towards higher levels of OPN compared to APD and saline 1 week controls ($p>0.05$). BSP levels were however had a tendency towards reduced levels in the HEBP treated group compared to APD and saline ($p>0.05$). Represented are the means of the measurements taken from the rats with the vertical bar representing the standard deviation.

Immunohistochemistry, one week results



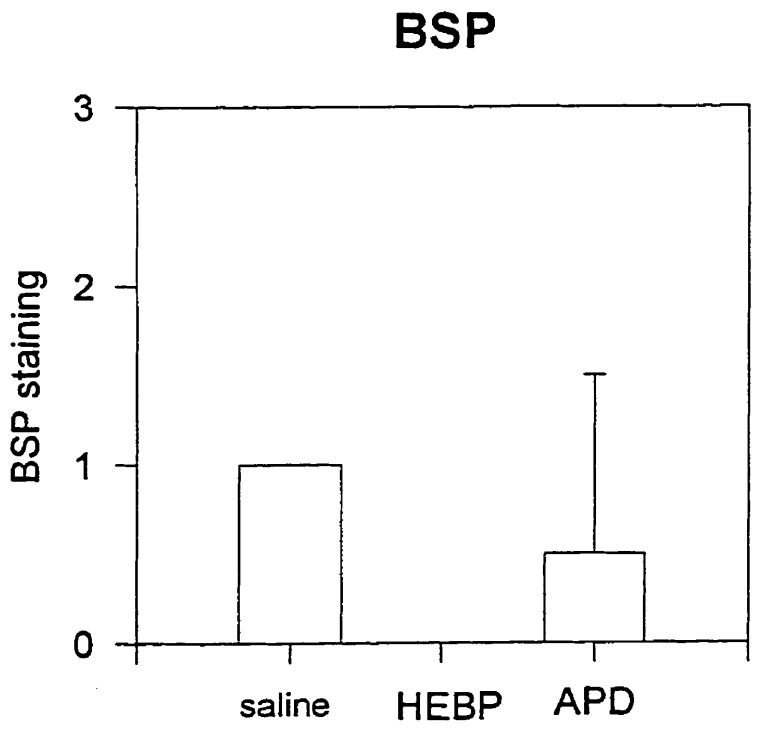
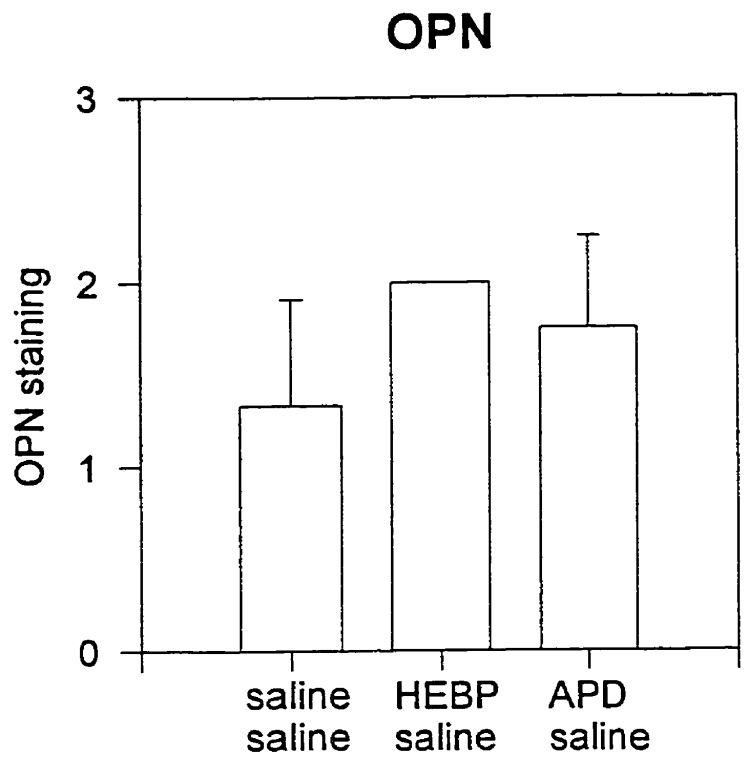


Figure II.8. **Two week results for levels of bone associated proteins BSP and OPN within the healing wound. determined by immunohistochemistry**

When the saline, HEBP and APD groups were allowed a second week for recovery from the drugs, levels of OPN and BSP returned to similar levels within all groups. There were no statistically significant differences between groups. Represented are the means of the measurements taken from rats, with the vertical bars representing the standard deviations.

Immunohistochemistry, two week results



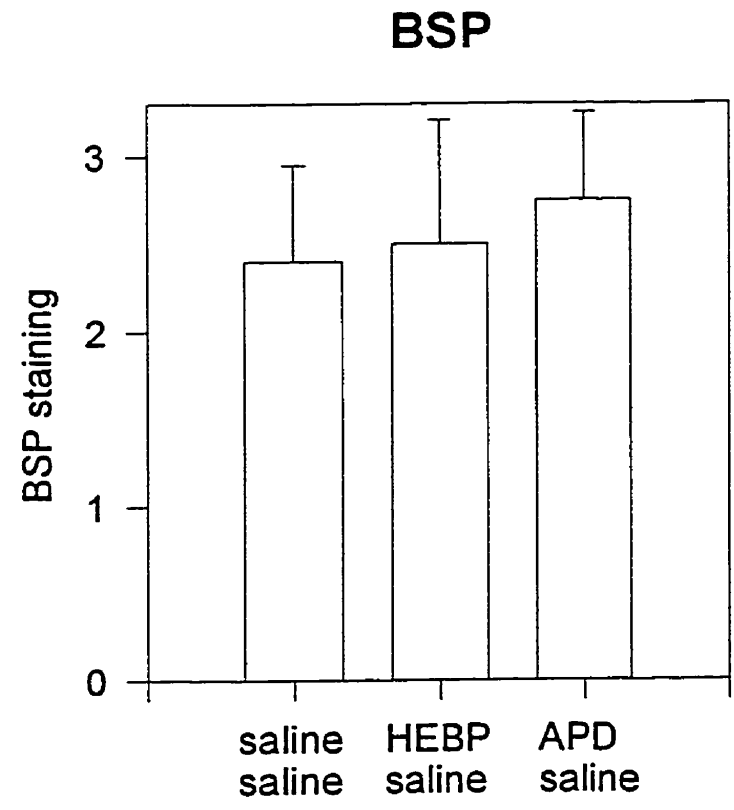


Figure II.9. **Level of proliferating cells within the healing wound, as determined by autoradiography of ^{3}H-Thymidine**

Treatment of rats with bisphosphonates HEBP and APD resulted in a significant decrease in cellular proliferation as compared to saline control after one week ($p=0.001$ and $p=0.02$ respectively). After a one week recovery period, all groups had significantly reduced from their respective levels of the previous week ($p<0.005$), and the HEBP as well as the APD groups were still significantly lower than two week saline controls ($p=0.001$ and $p=0.03$ respectively). There were no significant differences between bisphosphonate groups at either week. Represented are the means of the labeled cells per field taken from the five rats per group, with the vertical bars representing the standard deviations. The * represents a statistically significant difference from the respective saline control.

Total count of radio-labeled cells

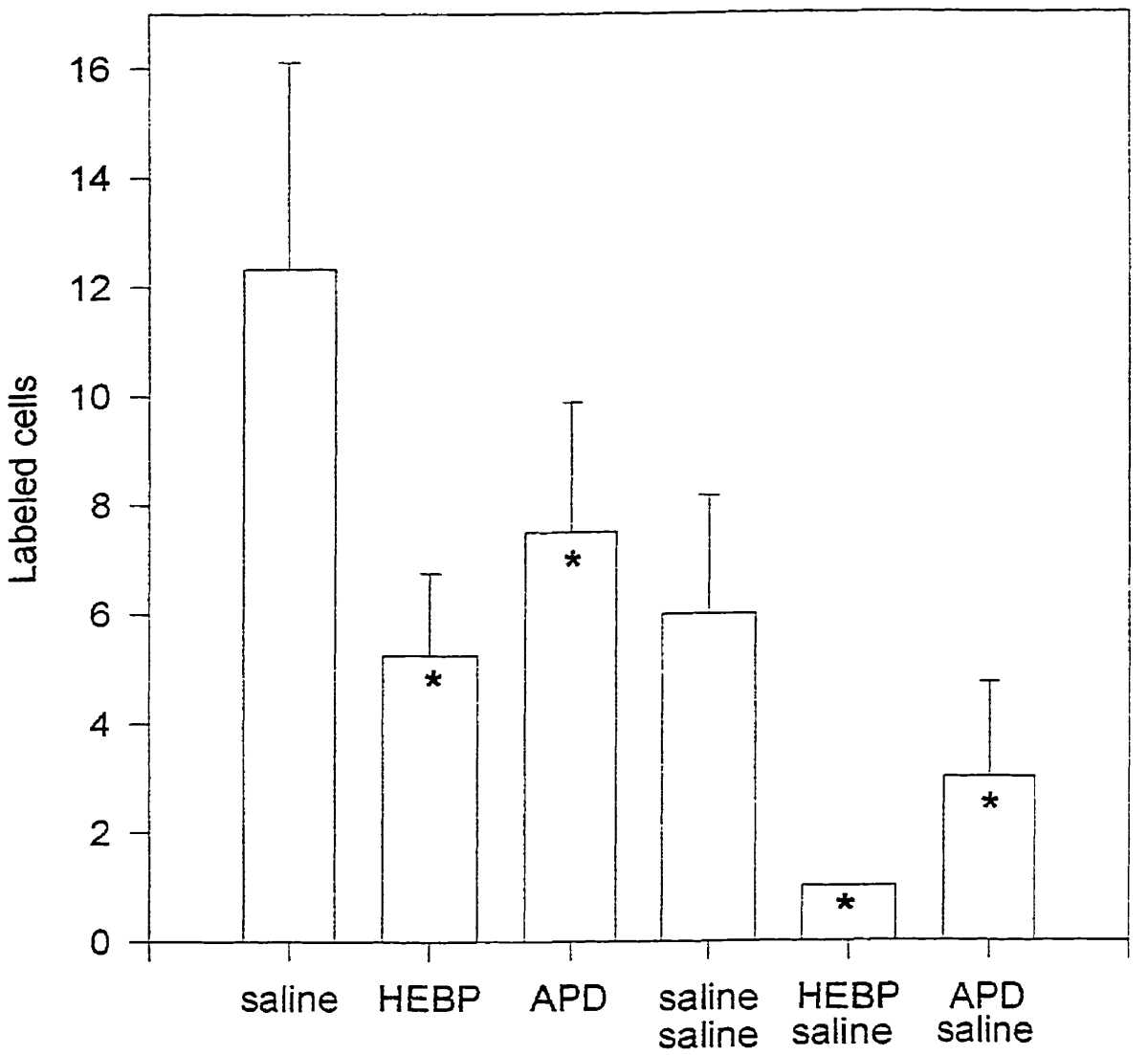
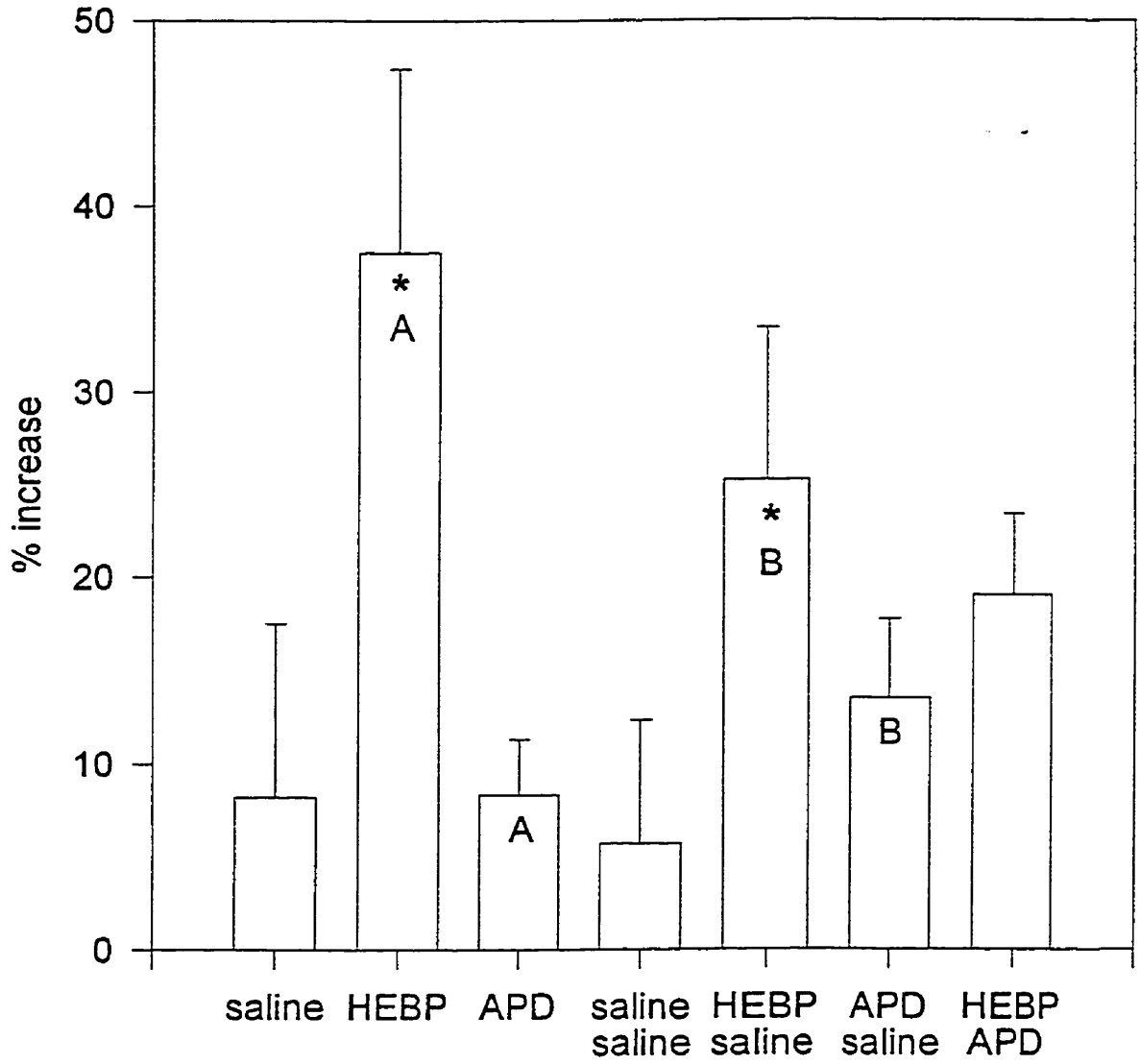


Figure II.10. Thickness of the calvaria

The percent increase in the rat calvaria thickness in presented. One week of HEBP treatment resulted in significant increase in calvarial thickness ($p<0.05$), of which 27% was lost during the recovery week ($p<0.05$). Administration of APD in the recovery week did not prevent this bone loss ($p=0.48$). Presented are the means of the rats per group with the vertical bars representing the standard deviation. The * represents a statistically significant difference from the respective saline control ($p<0.05$). Letters represent statistically significant differences between groups ($p<0.05$).

Percent increase in thickness of calvaria



Chapter III

Effects of Bisphosphonates on Non Wounded Bones

III.1. Introduction

Bisphosphonates are considered to be potent inhibitors of bone resorption. In light of this, the acceleration in wound closure observed in the rat calvarial model could in fact simply be due to the inhibitory effects on osteoclasts as opposed to an actual increase in bone matrix production. It was therefore proposed to determine if an increase in bone formation does indeed occur when the rats are treated with HEBP. This would indicate that HEBP induced stimulation of bone production would ultimately be responsible for the acceleration in closure of the wound as opposed to only the bisphosphonate's ability to inhibit bone resorption. Bone matrix production was demonstrated by injection of the rats with ^{3}H-proline, as it would be incorporated into newly produced collagen. Fluorochromes such as tetracycline and calcein were also used at different time points to analyze bone mineralization patterns and kinetics of mineralization. It has previously been observed in our laboratory that quantification of such labels in a healing wound is virtually impossible, since clear deposition lines are tremendously blurred due to plane of section problems. Accordingly I elected to quantify the effect of the bisphosphonates on bone production in non-wounded bones.

III.2. Materials and Methods

III.2.a. Animal Model

See section II.2.a.

III.2.b. Drug administration

The rats received daily subcutaneous injections of bisphosphonates as per the following schedule:

Table III.1. Experimental and control groups for assessment of non-wounded bones

GROUP	FIRST WEEK	SECOND WEEK
A	HEBP	
B	saline	
C	saline	saline
D	HEBP	saline
E	HEBP	APD
F	APD	saline

In order to label newly produced collagen, the rats in group A and B were injected intraperitoneally with ^{3}H-proline (2 μCi/g, specific activity 21 Ci/mmol) twenty-four hours apart on days three and four. To label mineralizing bone, all rats were injected intraperitoneally with tetracycline (25mg/kg) twenty-four hours apart on days five and six. In addition to the tetracycline, groups C-F received intraperitoneal injections of calcein green (10mg/kg) (Sun et al. 1992) twenty-four hours apart on days ten and eleven so that mineralization during recovery could be studied.

III.2.c. Tissue Preparation

The animals were sacrificed (see section II.2.c.) and their femurs and mandible were resected. The femurs were sectioned in half, length-wise, and the mandible one millimeter anterior to the first molar. This trimming was accomplished by using a low-speed saw (Buehler Ltd., USA) and a diamond wheel (Norton Co., USA). The right mandibles and femurs were fixed in preparation for demineralized sections and embedded in paraffin. The left mandibles and femurs were then fixed in preparation for undemineralized sections and embedded in plastic.

III. 2.c.i. Demineralized sections

See II.2.c.i. for protocol on embedding.

III.2.c.ii. Undemineralized sections

See II.2.c.ii. for protocol on embedding . This protocol was followed with the exception of 70% ethanol being used as a fixative instead of formalin so that the fluorochromes would not be leached out of the specimens.

III.2.d. Autoradiography

See section II.2.e for the protocol describing autoradiography. Measurement of osteoid production rate was to be done by measuring the distance between the double label of ^{3}H-proline. In certain specimens, the bands from the double labels were in such close proximity that measuring a distance between them was not possible due to overlap. The ^{3}H-proline labels were visualized as one wide band instead of two distinct bands in

many of these cases. In light of this, the total width of the bands was measured as well as the distance from the inner edge of the band to the outer edge of the bone, instead of measuring the distance between the two bands. This quantification was accomplished with light microscopy (Matallux 3, Leitz) and with the aid of the bone morphometry program from the Bioquant morphometric system (R&M Biometrics, Nashville).

III.2.e. Fluorochromes

The fluorochromes tetracycline and calcein green were used as markers of mineralized bone formation. Both dynamic and static parameters were assessed. For dynamic parameters, the distances between the total width of the double labels of fluorochromes were quantified and the intensity of fluorescence from the double labels was measured as the static parameter. The width of the double label was assessed instead of the distance between the double label bands for reasons outlined above for ^{3}H-proline (see section III.2.d.). Unstained sections of undemineralized specimens embedded in spurr acrylic were examined using a Leitz Metallux 3 microscope with a 100 watt mercury AC (HBO-100) light source. A 10x NPL fluotar oil immersion objective was used, as well as appropriate filter blocks. For visualization of tetracycline labels, a D filter was used (excitation 355-425nm, emission 460nm). An I2/3 filter was used to visualize the calcein labels (excitation 450-490nm, emission 515nm). The images were assessed on a Dage-MTI series 70 video television camera equipped with a Newvicon grade 1 draw tube and parameters were quantified using the bone morphometry program from the Bioquant morphometric system (R&M Biometrics, Nashville).

III.3. Results

III.3.a. Radiolabeled matrix

With the use of ^{3}H-proline, the amount of matrix or osteoid (largely collagen) produced was quantified in the rats receiving HEBP or saline during week one. The production of matrix in HEBP-treated rats was almost two-fold greater than that observed in saline controls ($p= 0.003$). This was the case whether the total width of the radiolabeled band was measured, or the distance from the band to the edge of the bone was quantified (figures III.1-III.4).

III.3.b. Fluorochromes

Fluorochromes were used in order to further assess altered patterns in bone mineralization resulting from bisphosphonate treatment, and also to assess changes in the kinetics of mineralization. In the absence of a recovery period, HEBP treatment resulted in significantly less tetracycline uptake ($p<0.05$) as compared to saline controls (figure III.5). This trend was maintained with the recovery period, but the results were highly variable, especially for the second week label of calcein green. It was not possible to establish any type of definite pattern in regards to the distance between fluorochrome bands, their width or their intensity.

III. 5. Legends, Figures and Tables

Figure III.1. **Total width of the ^{3}H-proline band in non-wounded femurs**

The total width of the band represents the amount of matrix (largely collagen) produced between days three and four (i.e. the time when the i.p. injections of radiolabeled marker were given). There was almost a two fold increase in the amount of matrix produced in rats treated with HEBP as compared to saline controls ($p= 0.003$). Represented are the means of the measurements taken from the three rats per group with the vertical lines representing the SD.

Total width of proline label in femurs

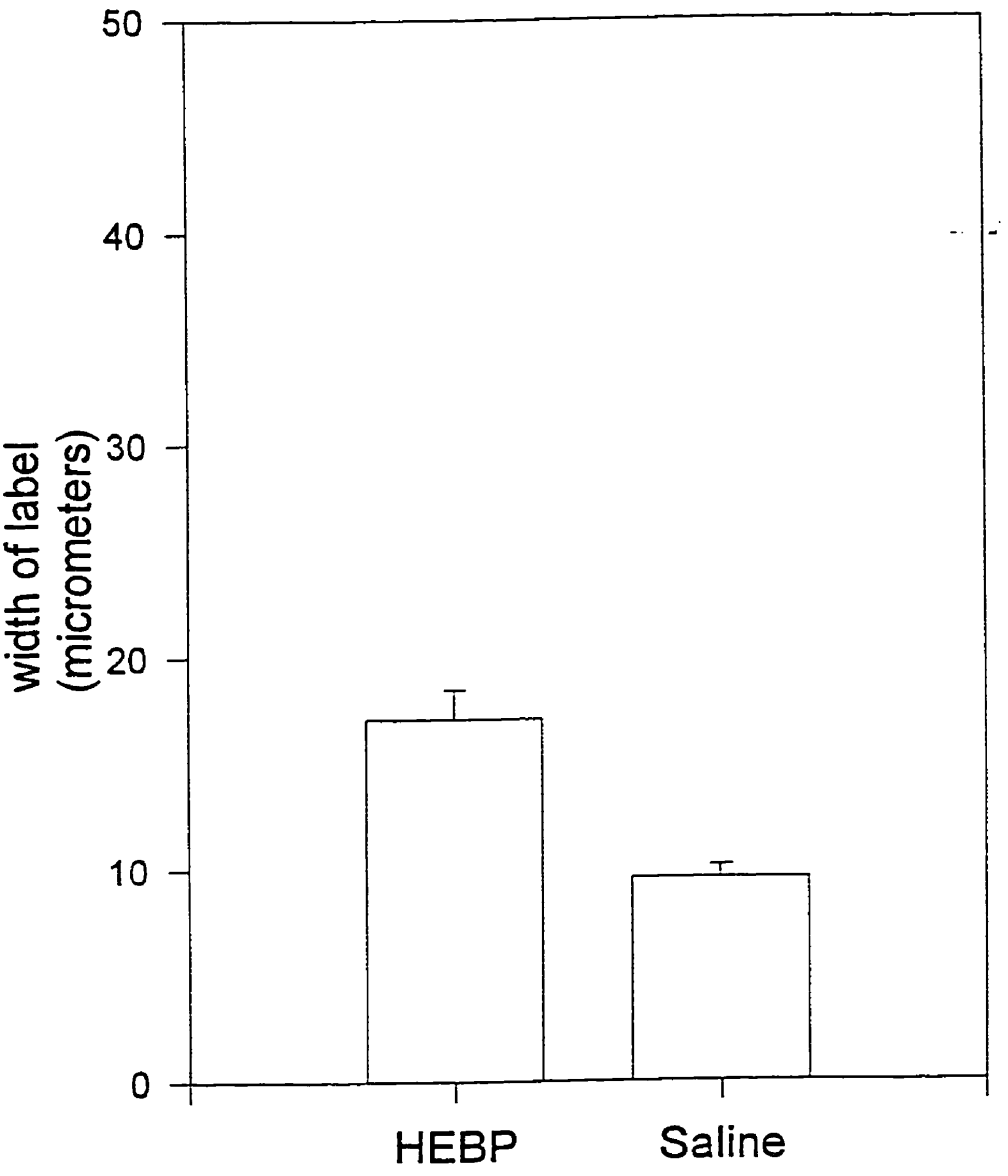
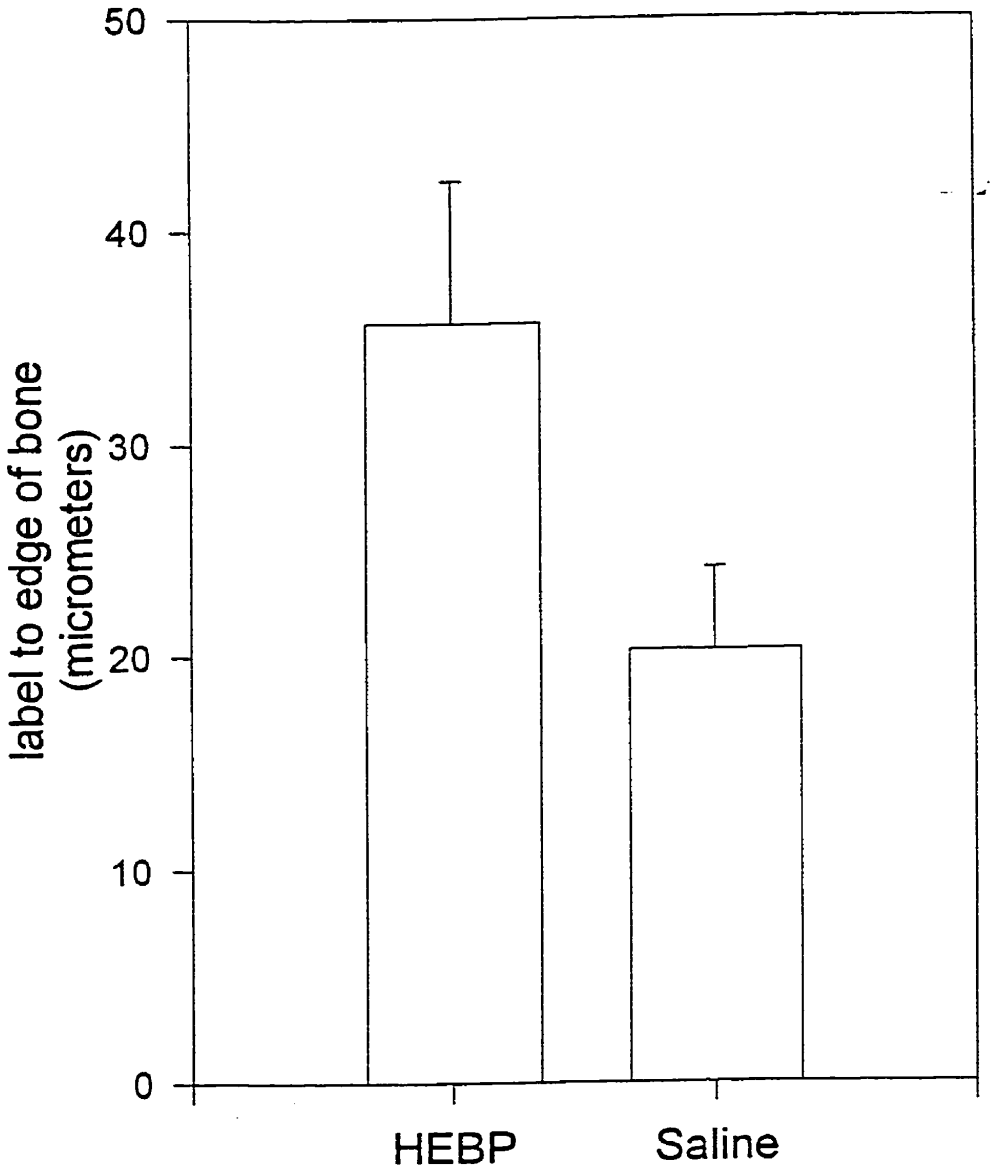


Figure III.2. Distance between the ^{3}H-proline band and the outer edge of the femur

The amount of matrix produced from the time of injection of ^{3}H-proline to the time of sacrifice on day seven is represented. There was almost a two fold increase in the amount of collagen produced in rats exposed to 1 week of HEBP compared to saline controls ($p= 0.03$). Presented are the means of the rats per group with the vertical bars representing the SD.

Proline label to edge of bone in femurs



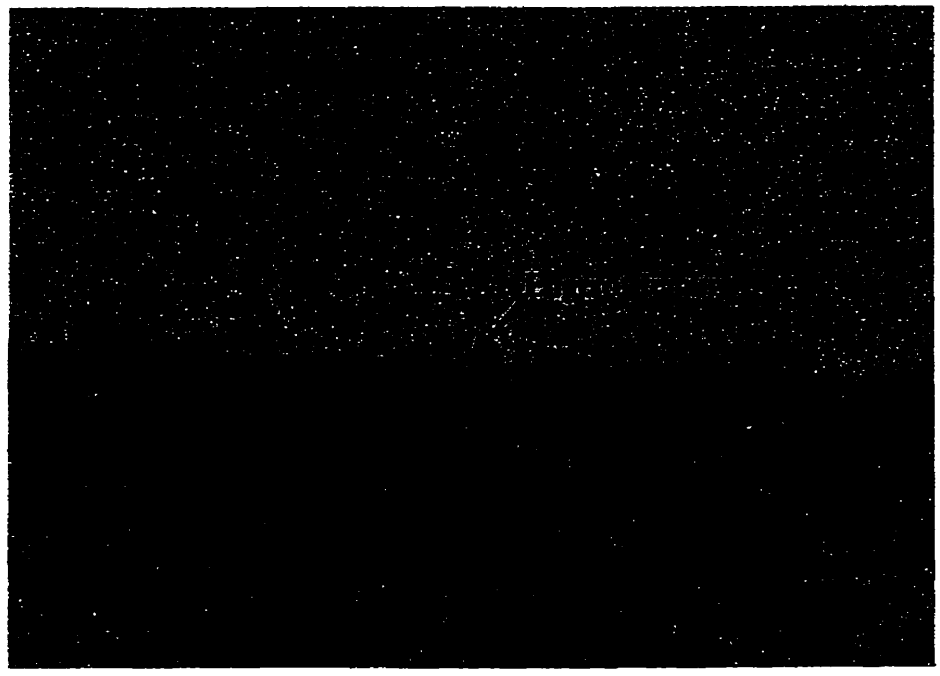


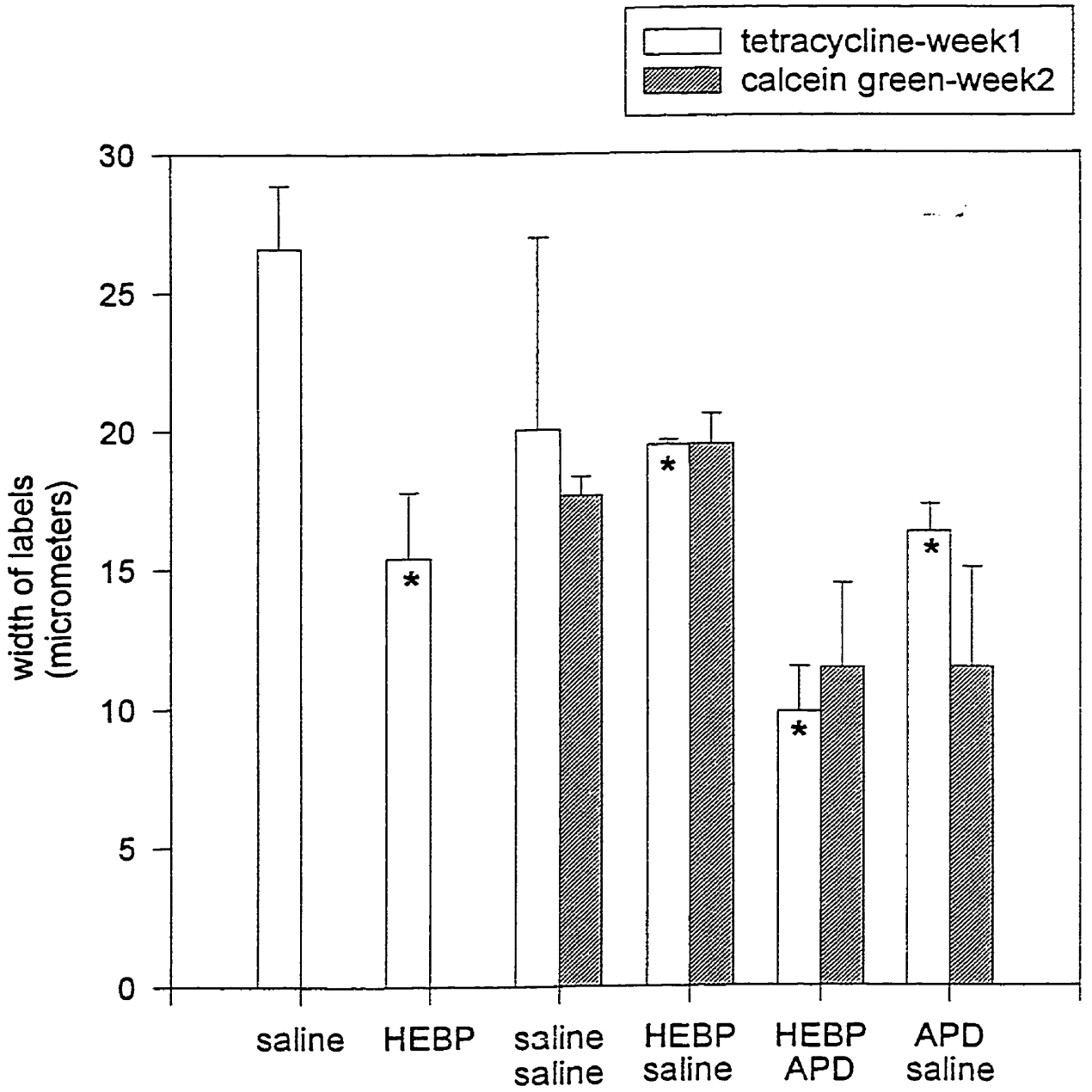
Figure III.4. Proline labeling of saline treated rat femur
H&E staining and autoradiography of a saline treated rat femur exposed to a double label of ^{3}H-proline on days three and four. (magnification 312x)

Figure III.5. Proline labeling of HEBP treated rat femur
H&E staining and autoradiography of an HEBP treated rat femur exposed to a double label of ^{3}H-proline on days three and four. (magnification 312x)

Figure III.5. Width of the fluorochrome labels in non-wounded femurs

Quantity of mineralizing bone is presented. Width of the tetracycline label from injections on days five and six and width of the calcein label from injections on days ten and eleven are from non wounded femurs. Presented are the means of the rats per group with the vertical bars representing the SD. The * represents a statistically significant difference from the saline control ($p<0.05$).

Fluorochrome labeling of non-wounded femurs



Chapter IV

Discussion

IV.1 Discussion

The purpose of this research project was to study calvarial wound healing under the influence of bisphosphonates. As alluded to earlier, data obtained from experiments carried out *in vitro* demonstrated an increase in bone matrix production in the CPO model when mineralization was inhibited with the use of HEBP (Goziotis et al. 1995). Subsequent mineralization of this matrix occurred following cessation of culture exposure to HEBP. It was therefore decided to assess effects of bisphosphonate treatment on bone wound healing following drug administration schedules developed *in vitro*.

IV. 2 Calvarial wound closure

Calvarial wound closure was assessed for all groups on the non-demineralized specimens, and based on these results, treatment groups were culled in order to focus on the significant ones for further assessment such as immunohistochemistry and autoradiography. Calvarial wound closure was assessed following seven days of treatment with the bisphosphonates HEBP and APD or saline (control) (groups 1,2,3). Acceleration of bone wound closure was limited to the group of rats treated with HEBP, as there was no statistically significant difference in closure as compared to saline control in rats treated with APD. This two-fold increase in closure is parallel to the previously reported (Lekic et al. 1997) two-fold increase in alveolar bone volume in rats treated with an identical dose of HEBP for one week. Similarly, I found that

calvarial thickness increased thirty eight percent in HEBP treated rats. Since bisphosphonates have two recognized modes of action (i.e. inhibition of bone mineralization and bone resorption), either one of these could have played a role in the acceleration of wound closure. *In vitro* data (Goziotis et al. 1995)would suggest that this burst of bone formation could be the result of, or is at least correlated with inhibition of bone mineralization that then appears to cause increased production of bone matrix. Results from these one week groups would tend to support this since the rats exposed to a mineralization inhibitory dose of HEBP had a two fold increase in wound closure whereas no increase, over saline control, was observed in rats receiving APD at a dose which inhibits bone resorption but supposedly has minor or no effects on mineralization. Hence, it appears that the acceleration of bone wound closure during this one-week exposure to HEBP could be related to its inhibitory effects on bone mineralization.

Previously published data obtained from *in vitro* studies (Tenenbaum et al. 1992) indicated that the bone matrix produced in presence of HEBP would mineralize once the drug was removed and such cultures were given time to recover from the mineralization-inhibitory effects of the bisphosphonate. Three parallel groups of rats (groups 4,5,6) therefore received the same drug regimen as the one week controls, but were allowed a second recovery week in the absence of bisphosphonate to permit the newly produced bone matrix to mineralize. As in the first week, the greatest amount of wound closure was observed in the HEBP-treated rats as compared to either APD or saline. This could indicate that the bone matrix produced during week one in the HEBP

treated rats was maintained for the most part during the recovery period. It can not be stated with total assurance that there was no loss of the newly formed matrix during the recovery week, as markers of bone formation were not used to quantify bone formation or resorption within the wound. However, the net result was that even more closure of the wound occurred as compared to week one. Indeed, one would expect further bone production during the recovery phase as we are dealing with a healing wound. Interestingly, the continued increase in wound closure during week two would initially seem to conflict with previous reports showing a 25% loss of newly formed matrix in the alveolar bone in rats recovering from an initial week of HEBP treatment (Lekic et al. 1997). However, in that study the massive production of bone matrix during the one week exposure to HEBP occurred outside of the "normal" envelope of bone typically occupied by the alveolar housing. This could be considered as "excessive" bone, which was likely getting little mechanical stimulation resulting in its 25% loss during the one-week recovery period. I would hypothesize that further loss of this excess bone would have occurred had the rats been followed for longer periods of time. On the other hand, loss of newly formed tissue within the wound was not apparent in this study, but this is a biologically different situation in that there is a wound which already has healing potential as seen in the saline controls. Bone is therefore being produced in an area where it would naturally occur, as opposed to stimulation of bone production beyond its normal anatomical peripheries.

Despite these issues, it was hypothesized originally that the partial loss of newly formed bone that was observed following cessation of HEBP treatment in earlier

studies (Lekic et al. 1997) might also occur in the calvarial wounds. To circumvent this possibility, it was rationalized to attempt inhibition of this predicted resorption that could occur in the wounds after cessation of HEBP treatment by subsequent treatment with APD. Notably, the dose of APD used ostensibly does not interfere with mineralization (Schenk et al. 1986) but should still inhibit resorption. The data show that although there appeared to be a trend that suggested greater wound closure in animals treated with APD during recovery, the differences seen were not statistically significant. There may be a number of reasons for this including the possibility that the null hypothesis was wrongly accepted as a reflection of sample size and power. However this could also be due, as discussed above, to the biological differences in a healing wound as compared to excessive bone produced outside the "normal" bony envelope, in that osteoclastic bone resorption is likely not a predominant factor in the healing wound resulting in minimal effect from APD treatment.

Treatment of rats with HEBP both in the first and second weeks did not significantly increase wound closure as compared to HEBP-saline treated rats. This would suggest that the stimulation of closure appears to be the result of a burst of bone matrix production in the first week as opposed to an endless and continuous stimulation of bone matrix production. This would correlate with *in vitro* effects of bisphosphonates on bone metabolism, in that twelve day cultures treated with HEBP had no increase in bone production over saline controls, but six day cultures exposed to HEBP had clear increases in bone–osteoid area (Goziotis et al. 1995). These *in vitro* and *in vivo* results would suggest that initially, osteoblasts may be induced to synthesize increased

volumes of bone matrix when exposed to HEBP, but over time, the osteoblasts ultimately make the same amount of matrix. Further studies of this question should focus on measurements of the actual amounts of bone formed per osteoblast in order to determine whether the findings produced here are parallel with those demonstrated *in vitro*.

IV. 3. Mineral/osteoid within the wound

Since it was hypothesized that HEBP's ability to accelerate wound closure was related to its inhibitory effects on mineralization, it was essential to confirm that this was actually occurring. Therefore, the relative amounts of osteoid and mineralized bone within the healing wounds were measured under the various experimental conditions. After one week of treatment with HEBP, 71% of the newly formed tissue within the wound was osteoid as compared to only 44% in saline control. This finding is consistent with the inhibitory effects of HEBP on mineralization, as most of the tissue produced during treatment with HEBP was in the form of bone matrix (osteoid). These results then largely support the previous findings showing an inverse relationship between mineralization and bone matrix formation. However, some mineralization of osteoid within the wound did occur with HEBP treatment, albeit in small amounts, suggesting that HEBP does not block mineralization *in vivo* totally. In fact, it is possible that similar phenomena may be occurring in patients and animals treated with other crystal poisons such as fluoride. Fluoride is also known to retard hydroxyapatite crystal growth but does not inhibit it completely (Lundy et al. 1986). In such situations, hyperosteoidosis also occurs and it is tempting to suggest that this is not simply relative

hyperosteoidosis but a manifestation of the phenomena described here. In any case, it seems clear that HEBP does appear to inhibit mineralization more effectively *in vitro* than *in vivo*. Notably, it has been shown in our laboratory (unpublished data) that HEBP is retained in the bone matrix even after cessation of treatment and following extensive extraction using guanidine and guanidine EDTA. Thus, it might even be surprising that following cessation of HEBP treatment, mineralization still ensues within the treated tissues. Moreover, previous *in vitro* data suggest that not only does mineralization occur in tissues that still harbor some HEBP, but that the mineral formed is actually denser than that found in control tissues (Torontali et al. 1994). Perhaps there is a threshold below which inhibition of mineralization will not occur and which may even stimulate mineral formation to some degree. These issues will be discussed again below.

Other findings showed that rats treated with APD for one week had a tendency towards greater amounts of mineralized tissue within the wound as compared to saline control, even though this was not significant. These data may be consistent with data obtained from *in vitro* studies showing that low dose APD induced an increase in mineral accumulation relative to APD free (control) bone-forming cultures (Tenenbaum et al. 1992). In the culture system used, it appeared that this was not related to an antiresorptive effect of APD, since there was no evidence for bone resorption in that model (CPO). Moreover, as indicated above, few or no osteoclasts were detectable histologically via either TRAP staining or visual assessment in any group and so perturbation of osteoclastic function may not have occurred to a significant degree in

any case. The actual mechanism behind this phenomenon of increased mineralization is not known.

When rats were allowed a one-week recovery period following an initial week of HEBP treatment, the osteoid to mineral ratios became similar to saline control. The large amounts of osteoid seen after one week of HEBP treatment therefore must have mineralized, resulting in a normal bone matrix to mineralized tissue percentage. It is noteworthy that the inhibitory effects of HEBP on mineralization appear to be reversible *in vivo* and that the large amounts of osteoid produced during exposure to HEBP do mineralize, as shown in previous *in vitro* (Tenenbaum et al. 1992) and *in vivo* studies (King et al. 1971).

The elevated levels of osteoid observed in the rats exposed to one week of HEBP did not return to the saline control levels when APD was administered in the recovery week. It would appear than, that mineralization of the newly produced osteoid was retarded somewhat by the subsequent treatment with the APD. This was unexpected, since the data obtained from previous studies carried out *in vitro* indicated that mineral accumulation actually increased in cultures exposed to APD. In addition to this, it would appear that APD might have stimulated mineralization when it was administered to rats for seven days (see discussion above). There is however an important difference between the rats treated with APD in the first week and the ones administered APD during the second week in that the latter were also exposed to HEBP in week one. It is possible that the inhibitory effects of the HEBP on mineralization were still pronounced

in the early part of week two, and mineralization of the large amounts of newly produced osteoid was delayed because of this, despite the presence of APD. Perhaps the percentage of osteoid to mineralized tissue had therefore simply not yet reached the levels of the saline control. In addition to this, the combination of both bisphosphonates, that is the presence of the HEBP which alters the dynamics of mineralization during week one followed by APD with its own suspected effects on mineralization (enhancement?), could have an unknown deleterious effects on mineralization of the massive amounts of osteoid produced in week one. Either way, I would suspect that the percentage of osteoid to mineralized tissue would have returned to similar levels as the saline controls had the rats been followed over more extended period of time than used here.

Although von Kossa staining was used to assess the effects of HEBP or APD on mineralization by studying bone/osteoid percentages, I also wanted to elucidate the kinetics of mineralization as influenced by either of these drugs. Hence, I chose to label mineralizing bone with a variety of fluorochromes at different time points. Unfortunately, the measurements obtained with this approach were difficult to interpret and were not always consistent with either the von Kossa staining assessments or the other data. There was some consistency in that less uptake in tetracycline did occur during the first week in HEBP treated rats as compared to saline control. This would suggest a reduction in mineralization occurred during this time period, similar to indications based on results from the von Kossa staining. However, the results from the calcein green labels during the recovery week did not appear to have any obvious

patterns. In an attempt to clarify these findings, in addition to measuring the distances between labels, I also measured intensity of labeling (data not shown) on the assumption that uptake of the fluorochromes may have been perturbed as well as rate of mineral formation, but even these findings were inconsistent. Perhaps retained HEBP in the matrix may have perturbed uptake of the fluorochromes such that the data obtained were not consistent with the morphometric findings. In any case, further study of this phenomenon is warranted.

IV.4. Calvarial thickness

An increase in calvarial thickness was observed following one week of HEBP treatment as compared to APD or saline groups. Twenty seven percent of this newly formed osteoid was lost during the recovery period, similar to that previously reported in an earlier study (Lekic et al. 1997). Similar results also occurred in a periodontal wound-healing model (Rajshankar et al. 1998) where bone morphogenic protein stimulated bone production well beyond the normal confinements of the alveolar bone, but where the excess bone was rapidly lost resulting in hard tissue limited to the normal alveolar bone envelope. In this study, the additional use of APD during the recovery week was used to prevent this anticipated loss of osteoid, but did not result in any significant decrease in its loss. However, it is conceivable that APD, at the dose used in this study, did not actually inhibit osteoclast-mediated resorption of the newly formed or pre-existing bone at all. Although this is possible, numerous investigations have shown that this dose is quite effective at inhibiting resorption (Sietsema et al. 1989; Schenk et

al. 1986) in the rat model. It is also possible that the resorption of newly formed bone seen here is not mediated by osteoclasts. In this regard, I did not observe any dramatic alterations in osteoclast numbers and in fact, osteoclast cells were very sparsely distributed in the tissues and sites examined. It has been suggested that osteoclasts require a mineralized bone substrate to be activated and that they will not resorb non-mineralized bone (Aaron et al. 1976; Irving 1963). Inasmuch as HEBP treatment induced the formation of substantial amounts of osteoid, osteoclasts might not have been activated or recruited to resorb the "excess" matrix, and so APD, an inhibitor of osteoclastic activity, did not effectively prevent involution of this excess bone. So this implies, but certainly does not prove, that this partial involution is a non-osteoclastic effect. Given this notion, it is conceivable that non-osteoclastic phagocytic cells such as monocytes and macrophages, cells that have demonstrable collagen phagocytic activity (Kahn et al. 1978; Mundy et al. 1977), could be resorbing the excess osteoid. However, it remains to be seen whether APD or HEBP have any effect on collagen phagocytosis and further whether the aforementioned cells are playing a role in involution at all. Further in this regard, it has been suggested that different tissues may occupy their own domains (McCulloch et al. 1983). Thus, quite apart from mechanical stimulation being required to maintain the extra bone formed during the experiments, it is also possible that in exerting hierarchy over their own domains, adjacent connective tissues are reinforcing the original anatomical relationships between themselves and the nearby bone. Indeed, this may be what is occurring in this study, since calvarial thickness was initially increased in the first week of HEBP treatment but then was reduced to a similar extent as seen with alveolar bone in earlier investigations (Lekic et

al. 1997). Thus, within the same bone (the calvaria), there was a reduction in "excess" thickness in non-wounded areas that might encroach upon adjacent soft connective tissue domains while within the wounds, there was no reduction in bone volume over the second week during recovery. The mechanisms for this phenomenon are not entirely clear. However, as an example, periodontal ligament soft connective tissues may protect their domains thereby maintaining periodontal ligament width by inhibiting in-growth of bone, probably by producing prostaglandins (Osigo et al. 1991). Similar mechanisms may be playing a role here.

IV. 5. ^{3}H-proline uptake

One of the central issues that required confirmation in this study was that HEBP treatment did not only induce a relative hyperosteoidosis (i.e. same total amount of bone/osteoid but just less mineralization) but that increased osteoid formation was actually occurring. To address this question, the rats were injected with the radiolabeled marker ^{3}H-proline on days three and four and they were sacrificed after seven days of continuous exposure to the bisphosphonate or saline. Matrix production was assessed in two bones, the mandible and the femur. In both bones, there was significantly (two-fold) more matrix (presumably collagen) produced per unit time when the rats were treated with HEBP as compared to saline control. The results from the measurements of the total width of the band indicate that greater amounts of matrix were being produced during day three and four in the HEBP treated rats. By measuring the distance between the radiolabeled band and the edge of the bone, it is evident that

this increased production of collagen in the HEBP treated group was maintained from day four to day seven. Since the predominant protein in bone matrix is collagen (Type I, 90% of total proteins) (Baron 1990), and given proline content of that protein, it would appear that when rats are administered HEBP, the increase in bone matrix production must be related largely to increased production of collagen. This would suggest that the acceleration in calvarial wound healing observed in the HEBP treated rats could be the direct result of an increase in bone matrix production, notably type I collagen, as predicted by *in vitro* studies (Goziotis et al. 1995).

IV. 6. Immunohistochemistry

As the non-collagenous proteins of bone such as BSP and OPN might play an important role in regulation of mineralization and bone cell differentiation, the presence and relative levels of these proteins were studied using immunohistochemical approaches. After one week of treatment with HEBP, there were elevated levels of OPN within the wound suggesting an increase in osteoblastic differentiation and/or osteoblastic activity, as OPN is expressed largely, although not solely by osteogenic cells (Denhardt et al. 1993). Even though this increase was two-fold in size, this was not a statistically significant difference, likely due to small sample size and the small number of outcomes (i.e. 2 in many groups). An obvious trend was however evident as seen with the two fold increase in level of proteins. Such an increase in osteoblastic activity, as suggested by elevated levels of OPN, would correlate with the observed elevated production of collagen seen in HEBP treated rats (see section III.3.a). The pattern of

OPN levels mirrors the amount of calvarial wound closure seen after one week of treatment (see section II.3.a). That is, when the OPN level was elevated in the one week controls, there was also the greatest amount of calvarial wound closure (HEBP group). On the other hand, when the OPN levels were low, there was also significantly less wound closure (APD and saline groups).

At one week, BSP was present in the APD and saline group, but there were no detectable levels of the protein in the HEBP group. BSP is thought to play a role in the mineralization of bone (Chen et al. 1991), and so the absence of this protein in the HEBP group might explain how HEBP inhibits mineralization, at least in part (see below), in addition to its purely physicochemical effects on mineral crystal formation.

When all three groups of rats were allowed a recovery period of one week, there were no significant differences between groups in regard to levels of either protein, and it would appear that both returned to "normal" levels during the recovery period. These results indicate that in addition to HEBP effects on mineralization itself, there may also be more direct effects on the osteoblasts themselves. In this regard, both BSP and OPN are produced by osteoblasts and their levels are either down-regulated or up-regulated respectively by HEBP. Thus, osteoblastic production of these proteins may be altered by HEBP. Alternatively, the number of osteoblasts might be affected with HEBP but this was not measured here. In addition, it is also conceivable that, at least for BSP, osteoblasts are still producing normal levels of the protein but that its retention in the matrix was inhibited by HEBP. This may be related to the fact that BSP binds avidly to

hydroxyapatite and since formation of such crystals was inhibited by HEBP, BSP might not be localized in the matrix. This is somewhat the proverbial "chicken and egg" argument since inhibition of BSP by HEBP might, as indicated above, inhibit BSP-mediated crystal formation. Further study of this using such methods as *in situ* hybridization or Northern analysis is needed to look at changes in expression of either BSP or OPN. In fact, previous studies in our laboratory do suggest that HEBP perturbs BSP message production (Lekic et al. 1997), and so further studies along these lines are warranted.

IV. 7. Mechanisms of action

In this study, it has been shown that the closure of rat calvarial wounds can be stimulated by the administration of HEBP. The treatment of rats with this bisphosphonate also resulted in numerous physiological and cellular events such as decreases in mineralization with an increase in bone matrix formation, stimulation of collagen production, and alteration in the levels of non-collagenous bone proteins OPN and BSP. Having discussed the general interpretation of the data so far, I have not yet addressed in detail issues pertaining to the possible mechanisms underlying the observed phenomena, and this will be done below.

As alluded to above, the pivotal focus of this investigation was to study the relationship between HEBP's supposed ability to reversibly inhibit mineralization thereby leading to increased bone formation (Goziotis et al. 1995). In order to explore this issue

further, it is necessary to understand the process of mineralization itself, but unfortunately, and despite much study, this is still a poorly understood process. Some of the proposed theories on mineralization will be described.

Matrix Vesicles

It has been proposed that matrix vesicles, membrane bound bodies that exocytose from the plasma membrane of osteoblasts into the extra-cellular matrix space, may mediate mineralization of bone (Anderson 1969; Bonnuci 1971). Once in this space, the matrix vesicles have the biochemical requirements leading to deposition of hydroxyapatite crystals in the inner side of their membrane (Boyan et al. 1989). This initial crystallization results in obliteration of the vesicle membrane leading to clusters of mineral crystals in the extra-cellular space, which conglomerate into a mineralized mass.

Collagen as a Nucleator

Another described mechanism for bone mineralization involves the collagen fibrils in the extra-cellular matrix (Glimcher 1959). With the use of electron microscopy, it has been shown that hydroxyapatite crystals are deposited within the gap regions of the collagen fibrils. It would appear that these gaps, either by themselves or with other components at these sites, result in loci for initiation of bone mineralization due to locally elevated levels of inorganic ions in the extracellular fluids (Weiner et al.1986; Glimcher 1959).

Non-Collagenous Proteins as Nucleators

As mentioned, collagen has been regarded as a nucleator of mineralization. However, when matrix proteins are extracted from the collagen, its ability to nucleate hydroxyapatite is lost (Termine et al. 1981). It has therefore been suggested that the collagen serves as a scaffold orienting crystal alignment, but other matrix components would be responsible to initiate the mineral deposition (Glimcher 1989). Such could include bone matrix molecules such as the phosphorylated sialoproteins osteopontin (OPN) and bone sialoprotein (BSP). OPN is considered an inhibitor of hydroxyapatite formation (Boskey et al. 1993) while BSP is thought to be a nucleator of hydroxyapatite (Goldberg et al. 1993). It has been demonstrated that, *in vitro*, there is a correlation between increased expression of BSP and an increase in formation of mineralized tissue (Ibaraki et al. 1992). This correlation in association with the presence of BSP in newly forming mineral crystals (Sodek et al. 1992) would suggest that BSP could play a role in the initial formation of hydroxyapatite.

Alkaline Phosphatase/Organic Phosphates

In vitro, synthesis of mineralized bone like tissue can only be achieved by supplementing tissue cultures with organic phosphates, in particular β-glycerophosphate (GP) (Tenenbaum 1981). Not only was mineralized bone found in cultures treated with GP, but also overall bone matrix synthesis was greater in unmineralized cultures than GP-treated (mineralized) cultures (Tenenbaum et al. 1989). It's also been reported that the addition of GP to the cultures results in a decrease in alkaline phosphatase (AP) activity in cells of osteogenic lineage (Tenenbaum et al.

1987;Tenenbaum et al. 1989). In addition to this, GP treatment may also cause a decrease in cellular proliferation (Tenenbaum et al. 1989). The role of organic phosphates *in vivo* is not clear, but GP appears to play an important role in the mineralization of bone matrix *in vitro*. Other related organic phosphates such as phosphoethanolamine may have a similar role *in vivo*. Indeed, it was on the basis of studies using GP, that the initial hypothesis regarding HEBP's potential to regulate matrix synthesis was developed.

On the basis of these mineralization theories, it is hard to conceive how bisphosphonates and their inhibitory properties on bone mineralization via the physico-chemical nature of their action can alter such events as collagen production. How exactly can osteoid producing osteoblasts be affected by events governed at the mineralization front, 20 microns or so away, via physico-chemical blockage of crystal growth? It would be much more plausible that the observed physiological and cellular alterations in events associated with HEBP administration be the result of a biological mode of action of the bisphosphonate directly on osteoblasts as opposed to solely a physico-chemical one.

One possible biological mode of action of the HEBP could be related to its effects on non-collagenous proteins (NCP) due to their possible role in mineralization, as previously mentioned. The treatment of rats with HEBP during week one resulted in elevated levels of osteoid production and low levels of mineralized tissue within the wound. In association with this, elevated levels of OPN were detected within these

tissues as well as virtually non-existent levels of BSP. The inhibitory effects of HEBP on mineralization could therefore be mediated through down regulation of BSP expression while its effects on increased matrix production could be via up-regulation of OPN expression. Based on the previously mentioned theories on mineralization, there could be a number of mechanisms simultaneously involved in mineralization, which would explain why some mineralization did occur despite BSP levels being eradicated by the presence of HEBP.

The blockage of mineralization by HEBP through physico-chemical interactions with crystal growth is not being downplayed. To the contrary, this could be the route through which a cellular mode of action is being brought about. By blocking crystallization of hydroxyapatite, it would be expected that HEBP treatment could perturb local levels of free ions such as calcium and phosphorous. Calcium is an important regulator of physiologic and cellular processes, both intracellular (Berridge 1985), and extracellular (Brown 1991). It has been demonstrated that there is a cell surface calcium sensitive receptor which allows cells to respond to minute changes in the local calcium concentrations (Brown 1993). In addition to this, it has been shown that the calcium receptor is expressed by various cells in the bone marrow system (House et al. 1997). Perhaps this would allow for these bone cells to respond to local changes in calcium concentration, leading to possible regulation of differentiation or function of cellular activity which in turn would influence bone turnover and bone wound healing. This could perhaps play a role in the altered cellular activities noticed

in this study, giving rise to key biological alterations involved in acceleration of wound healing.

One cannot dismiss the possibility that the acceleration in calvarial bone wound healing observed is totally independent of the inhibitory effects of HEBP on mineralization and is in fact a serendipitous finding. The bisphosphonate could be causing an increase in collagen production through a mechanism unassociated to bone mineralization, but the two could simply be occurring simultaneously at the dose of HEBP that was used. Indeed, inhibition of bone mineralization did occur with the dosage of HEBP administered, but an unrelated cellular action could have occurred simultaneous to this, which could be the true cause of the accelerated production of osteoid leading to increased bone wound healing. For example, HEBP treatment reduced cellular proliferation (which could be indicative of terminal differentiation) and increased osteoid production phenomena, which are similar to the *in vitro* patterns observed with the addition or removal of GP to bone cell and tissue cultures (Tenenbaum et al. 1989). Perhaps the treatment of rats with HEBP altered serum levels of organic phosphates such as phosphoethanolamine resulting in similar findings to what occurs *in vitro* when organic phosphate levels are changed. However, given that the *in vitro* effects of HEBP on mineralization and bone matrix production were reproduced in this *in vivo* model, it would suggest a direct and local effect that is independent of systemic modulation. Further along these lines, our laboratory has shown that biologically available organic phosphates such as phosphoethanolamine do induce mineralization of

bone *in vitro*. Given that such organic phosphates are biologically available, it is conceivable, as alluded to above, they might play some role in regulation of bone cell metabolism *in vivo*. Although it is not yet known how organic phosphates regulate osteoblastic function and mineralization, it is plausible that HEBP directly modulates those effects, and in this case, inhibits them.

Thus, although bisphosphonates have been perceived historically as inhibitors of bone resorption and mineralization, their effects may be broader than their accepted mode of action in blocking mineralization through a physico-chemical interaction with hydroxyapatite crystals (Fleisch 1968). In light of the body of evidence in this study, it is conceivable that bisphosphonates have a cellular mode of action. The evidence for increased collagen production, altered levels of NCPs and changes in cell proliferation rate suggests a direct or indirect (possibly through the calcium receptor) cellular action of the bisphosphonate.

IV.8. Clinical impact

One can only begin to speculate on the impact of the adjunctive use of bisphosphonates in clinical situations requiring bone wound healing. It's clinical application should be aimed at stimulation of bone healing in circumstances where the bone already does heal slowly on its own, but the desired outcome would be a reduced healing time period. This is based on previous *in vitro* data (Goziotis et al. 1995) and evidence gathered here showing that HEBP stimulates a burst of osteoid production which tapers off gradually

as opposed to continuous accelerated production of osteoid, therefore ultimately leading to the same quantity of bone/osteoid being produced as controls, but at a much increased rate. Hence, it's use might not be intended for stimulation of healing for conditions that would not heal on their own, as in so called critical size wounds, but further study of this is needed. Foreseeable applications could therefore include shortening the time required for implanted prostheses to osseointegrate, or to accelerate the healing period of fractured bones or bone grafts.

One can anticipate potential drawbacks to stimulation of bone formation with the use of bisphosphonates. Even though initial histological data gathered here and in previous *in vitro* work (unpublished data from our lab) suggest that the bone produced as a result of the HEBP treatment appears to be normal, the ultimate mechanical properties of such bone are unknown. Will it be able to function under load? Will it be able to support an osseointegrated implant? One of the goals of bisphosphonate therapy in the field of osseointegration would be acceleration of the integration period. In addition to these concerns, would final osseointegration actually be hastened given that the initial tissue formed (i.e. osteoid) is not mineralized, thus requiring a waiting period for proper mineralization and maturation of this tissue. Further studies in these areas will be required before the transition to clinical applications can be made.

Even though systemically administered bisphosphonates have a tendency to accumulate preferentially in areas of increased bone formation, such as wounded bones (Fogelman et al. 1981), potential downfalls such as questionable long term effects on the overall

skeleton, unknown effects on growing bones and poor gastro-intestinal absorption would suggest potential benefits from local delivery of the bisphosphonate. In order to circumvent these pitfalls, future studies should investigate the development of a local delivery system, thus potentially benefiting from ideal local drug concentration, reduced systemic side effects, and elimination of need for patient compliance. The challenge will be in developing a system that initially delivers a high pulse dose of HEBP to then taper off resulting in an absence of drug in order to allow for a drug free recovery period. A similar rat-wounding model as employed in this study would be adequate for such an experiment. In order to investigate the possible application of bisphosphonates in the field of osseointegration, a rat model could be used in which miniature dental implants would be surgically placed in their femur and a reverse torque test be performed at different time points to determine if osseointegration is accelerated with HEBP treatment.

It is evident from the foregoing that there is great potential for clinical exploitation of the findings reported in this study. However, as indicated above, it is also noteworthy that there is as yet much to learn regarding the mechanisms of action of HEBP. Indeed, not only would information pertaining to HEBP mechanisms of action be useful in its own right, but such findings may lead to more useful insights regarding the mechanisms of mineralization itself. Thus, there is an obvious need to develop strategies to investigate regulation of organic phosphate and HEBP mediated effects on mineralization and matrix synthesis. Some intriguing data are already available from

the CPO model (Dr. Peter Fritz, personal communication). These data show that although GP obviously down-regulates production of collagen, message RNA for collagen is unaltered. This suggests post-translational and possibly even post-transcriptional effects of GP. Inasmuch as HEBP seems to modulate organic phosphate effects, at least *in vitro*, its effects may also be taking place at either or both of the post-translational or post-transcriptional level. If this were the case, collagen packaging and secretion events may be targeted by GP with or without HEBP while actual synthesis is not. In any event, much more investigation is required to elucidate HEBP's effect on bone formation.

Chapter V

Summary and Conclusions

V Summary and Conclusions

On a daily basis, clinicians are faced with various situations involving bone healing, from fractured bones to surgical placement of dental implants or joint prostheses. With this study, it has been shown that the process of bone healing can be accelerated with the adjunctive use of bisphosphonates, as shown in a previous pilot study (Tenenbaum et al. 1991). By using a rat calvarial wound model, it was possible to examine the effects of the drugs on the rate of bone wound healing as well as various parameters relevant to the mode of action of these drugs on bone physiology. This effect on bone healing appears to be unique to HEBP, and appears to be related its inhibitory action on bone mineralization. This inhibition of bone mineralization associated with increased production of osteoid appears to be reversible as the mineralization associated parameters that were examined returned to normal following cessation of HEBP. It was demonstrated that a high pulse dose of HEBP induced a two-fold increase in collagen synthesis based on ^{3}H-proline labeling, suggesting that the increase in rate of calvarial wound closure was due to stimulated osteoid production. The adjunctive use of APD during the recovery period from HEBP treatment did not produce a significant increase in bone wound closure.

It can only be speculated as to the mode of action of this bisphosphonate and the link between its inhibitory action on bone mineralization and its stimulatory action on osteoid production. Regardless of the underlying mechanism, the observed increase in

bone volume production could have significant clinical impact in many fields of medicine and dentistry.

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