EFFECT OF C-TYPE NATRIURETIC PEPTIDE (CNP) AND ESTRADIOL ON CULTURED HUMAN OSTEOBLAST CELLS (SaOS-2) AND FETAL MOUSE FEMURS AND TIBIAE

Shoba Ranganathan

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The mechanism of bone formation and development is not known. Estrogen is thought to have an important effect on bone. however. many other factors are also involved. It is well known that atrial natriuretic peptides are involved in blood pressure control and body fluid homeostasis. Recent evidence suggests that brain type natriuretic peptide (BNP) plays an important role in skeletal growth. In the present study. we examined the effect of C-type natriuretic peptide (CNP) and estradiol in cultured human osteoblastic cells and on fetal mouse femurs and tibiae.

The human osteosarcoma SaOS-2 cell line was cultured and subjected to various doses of estrogen (10^{-18} to 10^{-9} M) and CNP (10^{-10} to 10^{-7} M). Estradiol significantly increased ($p<0.05$) cell proliferation and alkaline phosphatase activity. The effect of CNP on this cell line. however. was not pronounced.

Femurs and tibiae from 17 gestational day (GD) B6 mice were isolated and cultured in minimal essential media supplemented with ascorbic acid. l-glutamine and sodium glycerophosphate for 8 days. On day 0. 4 and 8. bones were photographed and their length and area determined. Comparison was made between CNP-treated and vehicle-treated control bones. Data show that CNP-treated femurs and tibiae grew about 75% more than controls ($p<0.05$). The lengths of estradiol-treated bones were not different from controls.

Our results show that CNP does not have a profound effect on osteoblasts in the adult bone. whereas estrogen influences proliferation and alkaline phosphatase activity of these cells. The present study further indicates that there is a possible role for CNP in skeletal growth in the mouse during the late prenatal period and that estrogen alone may not be important in bone growth during this period of development.

*For my parents, who have
always given me their
guidance, support, enthusiasm,
and love.*

My parents have always guided me to be independent and encouraged me to achieve all my hopes and aspirations. I am grateful to them for their support and generosity. Their love and patience have been invaluable. I also thank Adam Bruce who has shown untiring patience and support, for reminding me of my priorities and keeping things in perspective.

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LIST OF ABBREVIATIONS

ALP .... alkaline phosphatase

ANP .... atrial natriuretic peptide

BCIP .... 5-bromo-4-chloro-3-indolyl phosphate

BNP .... brain natriuretic peptide

cGMP .... cyclic Guanosine MonoPhosphate

CNP .... C-type natriuretic peptide

CS .... calf serum

DEPC .... diethyl-pyrocarbonate

DNA .... deoxyribonucleic acid

EDTA .... ethylenediaminetetraacetic acid

FCS .... fetal calf serum

MOPS .... 3-(N-morpholino) propanesulfonic acid

mRNA .... messenger ribonucleic acid

NBT .... nitroblue tetrazolium

NPRA (GC-A) .... natriuretic peptide receptor A (guanalyl cyclase A)

NPRB (GC-B) .... natriuretic peptide receptor B (guanalyl cyclase A)

NPRC .... natriuretic peptide receptor C

OD .... optical density

PBS .... phosphate buffered saline

PCA .... perchloric acid

PCR .... polymerase chain reaction

PTH .... parathyroid hormone

RNA .... ribonucleic acid

RT-PCR .... reverse transcriptase polymerase chain reaction

SaOS-2 .... human osteosarcomic osteoblast-type cell line

Chapter 1

Introduction

Bone remodelling not only affects the growth of bones, but also the deterioration or general lack of growth, which may result in serious medical conditions where quality of life is threatened. The elucidation of the mechanism of action of bone formation and resorption may provide insight into combating conditions such as osteoporosis and skeletal dysplasias. While bone development and formation has been researched extensively the exact mechanisms involved are still unknown. Estrogen has already been established as an important hormone in bone pathophysiology. There has been new information and developments regarding the relationship between natriuretic peptides and bone development. In order to facilitate the discussion of the mechanism of bone formation, it is necessary to summarize the current status of research in this field.

1.1 BONE

1.1.1 Histology of Bone

Bone is a specialized form of connective tissue that is composed of cells and an organic extracellular matrix made up of proteoglycan ground substance and collagen fibres. The extracellular matrix, the mineral component of bone, is primarily composed of calcium hydroxyapatite crystals, whereas the fibrous component is primarily Type I collagen (Burkitt et al., 1993). At a cellular level, bone is composed of osteoclasts and a heterogeneous population of osteoblasts derived from the stromal cell system (Owen, 1985): mature osteoblasts, osteocytes and bone lining cells which cover bone surfaces. Mature osteoblasts are cuboidal cells that have a single defined nucleus and well-developed endoplasmic reticulum and golgi apparatus (Figure 1-1.A). They are involved in the synthesis and secretion of matrix proteins that are incorporated into the skeleton (Kleerekoper, 1996) including Type I collagen, osteocalcin, osteopontin, bone

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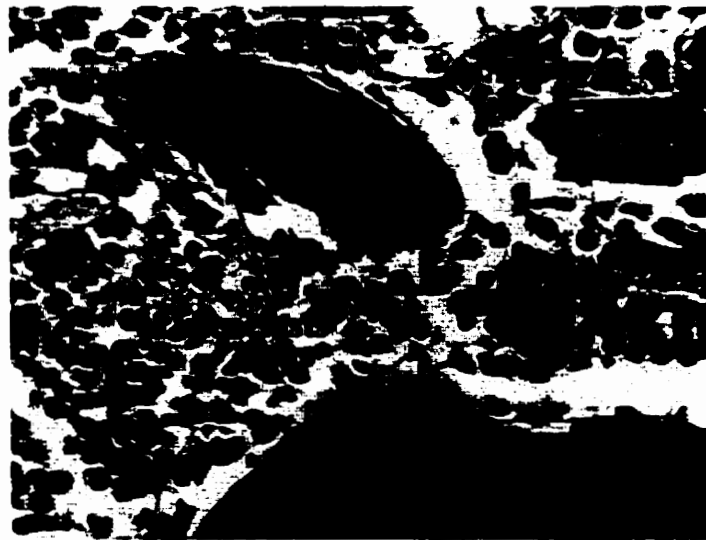
Figure 1-1: (A) An active layer of osteoblasts is present between the osteoid (stained red) and the mineralized bone (stained blue). *H & E stain 800x magnification..* (B) Large multinucleate cells called osteoclasts are often seen lying in bony depressions called lacunae. The aspect of the osteoclast adjacent to bone (stained blue) is characterised by a ruffled border. *H & E stain 800x magnification..*

sialoproteins, and osteonectin (Mundy, 1999). Mature osteoblasts are also known to secrete growth factors, such as transforming growth factor β and bone morphogenetic proteins, which are stored within bone matrix (Hauschka et al, 1986). Osteoblasts also produce factors that are vital in mineralizing the newly formed bone matrix. Once the osteoblasts have successfully synthesised bone matrix, they are incorporated into that matrix and eventually become osteocytes. Located in lacunae, situated between consecutive lamellae, these osteocytes are actively involved with the maintenance of the bony matrix and their death is followed by resorption of this matrix (Junquiera et al, 1995). Bone lining cells have been hypothesized to be osteocytes that are present on the bony surface. They have been implicated as accessory cells in the bone resorption process (Rodan et al, 1981; McSheehy et al, 1986; Mundy, 1999). The bone cells that are responsible for the resorptive processes associated with remodelling of bone are the osteoclasts (Burkitt et al, 1993). Osteoclasts are large multinucleated cells that are derived from the same precursors that give rise to the monocyte-macrophage system (Figure 1-1.B). They are found on the surface of bone embedded in the bone lining cells. When exposed to resorbing agents, the bone lining cells retract allowing the osteoclasts to integrate themselves into that area. They morphologically change by forming a ruffled border in order to resorb the bone at the exposed surface (Jones et al, 1985). Non-osteogenic cells such as adipocytes, vascular endothelial cells, and stromal cells are abundantly present in the bone marrow, and are also considered to be involved in the regulation of bone function (Recker et al, 1992).

1.1.2 Osseous Tissue

Each bone in the skeletal system contains different proportions of two basic types of osseous tissue: compact and cancellous bone (Figure 1-2.A). Compact, or dense bone comprises approximately 85% of the total skeleton. With a smooth and homogenous appearance, it is primarily found within the shafts of the long bones (Figure 1-2.B). Although it appears to be very dense, it houses a network of canals that serve as ducts for nerves, blood vessels, and lymphatics. The structural unit of compact bone is the Haversian system, or osteon, which is made up of lamellae, hollow cylindrical structures of collagen fibres, that are concentrically organized around a vascular canal. This central or Haversian canal contains small blood vessels and nerve fibres that supply the cells of the osteon. Volkmann's canals run perpendicular to and connect the vascular and nerve supply from Haversian canals and the medullary cavity to the periosteum, a double-layered membrane of connective tissue and osteogenic cells (Marieb, 1995). The volume of this type of bone is regulated by the formation of periosteal bone, remodelling within the Haversian systems, and by endosteal bone resorption. Decrease in compact bone mass is a major predisposing factor for fractures in hip and wrist. Cancellous, or spongy, bone makes up the remaining 15% of the skeletal system and is comprised of trabeculae only a few layers thick, with irregularly arranged lamellae and osteocytes (Figure 1-2.B). These small flat pieces of bone form a complex network with numerous interconnecting cavities. Since there are no Haversian systems present, the nutrients reach the osteocytes from the marrow spaces between the trabeculae via diffusion (Marieb, 1995). Decrease in cancellous bone mass is primarily through osteolytic disease caused by malignant cells that lodge into the marrow cavity. These malignant cells stimulate adjacent osteoclasts
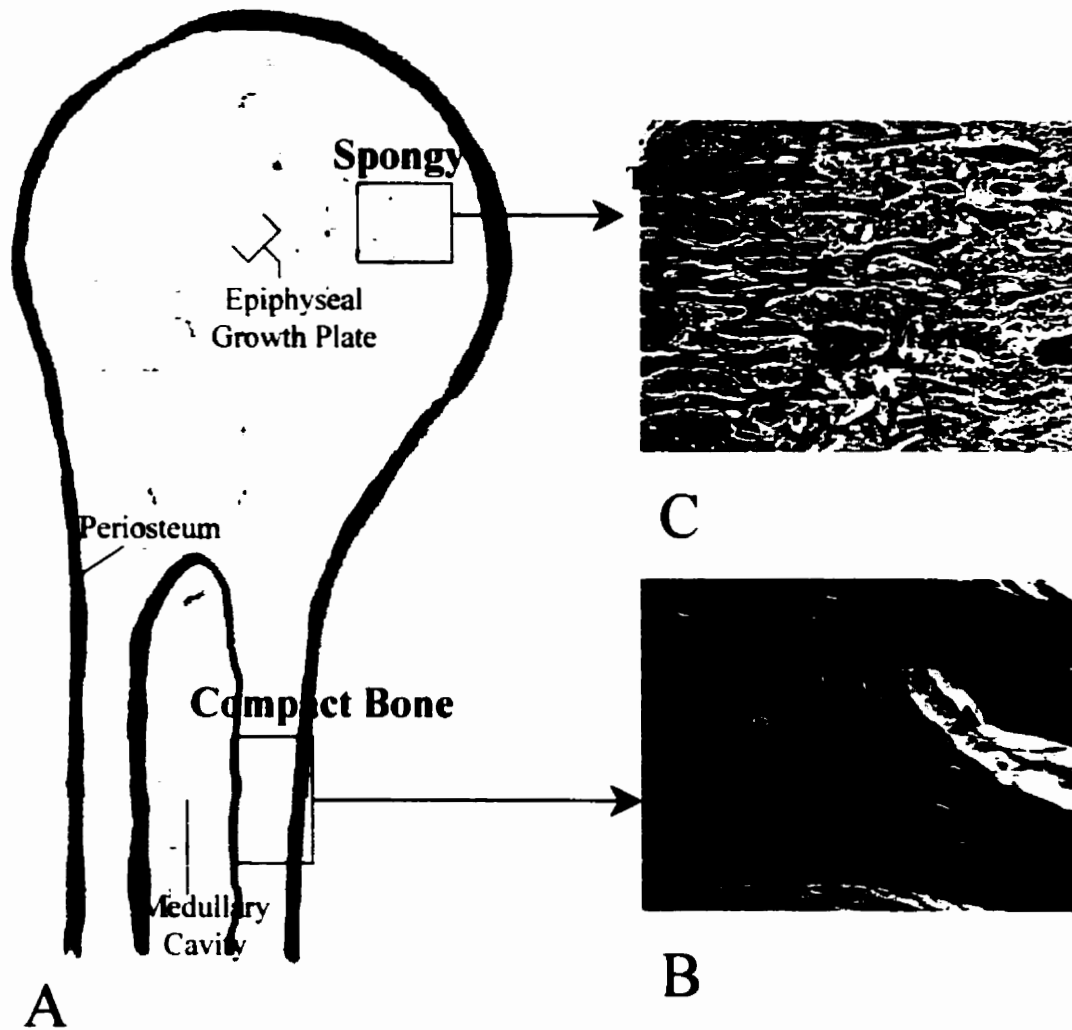
Figure 1-2: (A) Schematic diagram of the structure of an adult long bone indicating regions of compact and spongy bone. *H & E stain. 200x magnification* (B) Histological section of compact bone. (C) Histological section of cancellous bone. *H & E stain. 80x magnification.*

on trabecular plates and endosteal surfaces of cortical bone causing bone to be reabsorbed (Mundy et al.. 1974ab). Complete perforation and fragmentation of trabeculae is a cause of decreased cancellous bone loss due to aging (Parfitt. 1983: Kleerekoper. 1985).

1.1.3 Classification of Bone

Bones are found in a variety of sizes and shapes. which are dependent on the structural function of the bone in the body. There are primarily four classifications of bones: long. short. flat. and irregular. Long bones consist of an elongated shaft. diaphysis. with two terminal ends. epiphyses. The diaphysis is constructed of a thick collar of compact bone that surrounds a marrow cavity and covered on its outer surface is covered by periosteum. The epiphyses. also covered by periosteum. contain cancellous bone in the interior. Long bones comprise all the bones of the limbs except the patella. carpals. and tarsals which are classified as short bones. These cube-like short bones contain mostly cancellous bone. but are covered with a thin layer of compact bone. Flat bones are thin and slightly curved bones found in the thorax and the skull. They are comprised of two compact bone surfaces with a layer of cancellous bone between them. Irregular bones. such as the vertebrae and pelvic bones. have complicated shapes and do not fit any of the preceding categories. They usually are comprised mainly of cancellous bone enclosed by thin layers of compact bone.

1.1.4 Ossification

Ossification. the process of bone formation. occurs through two different mechanisms: membranous and endochondral ossification (Howell et al.. 1992).

Membranous bone formation occurs when mesenchymal cells condense and gradually differentiate into osteoblasts. These osteoblasts then produce an extracellular matrix which enlarges and mineralizes, ultimately replacing all of the mesenchyme (Mundy, 1999). This occurs in most of the craniofacial bones, such as the vault of the skull, the maxilla, and most of the mandible. Endochondral ossification involves the sequential formation and degradation of cartilaginous structures that serve as models for the developing bones. These hyaline cartilage structures, which are gradually converted to bone, serve as a temporary skeleton in the embryo and are broken down as ossification proceeds. A bone collar forms around shaft of hyaline cartilage leading to the calcification of the cartilage. A periosteal bud invades the internal cavities and spongy bone forms. This type of bone contains abundant vasculature, as vascular invasion is necessary for endochondral bone formation to occur (Recker, 1992). This is the process involved in the formation of the vertebrae, pelvis, bones in the base of the skull, and long bones (Iannotti, 1990, Suda et al., 1998, Recker, 1992, Howell et al., 1992).

1.1.5 Bone Development and Growth

Bone tissue begins to develop around six weeks in the human embryo. Most of the skeleton by this stage is constructed entirely from fibrous membranes and hyaline cartilage. In long bones, the diaphysis is the primary ossification centre which begins endochondral ossification around this time. The secondary ossification sites in the epiphyses appear shortly before or after birth. In bones formed by membranous ossification, ossification begins around the eighth week of development and continues until shortly after birth. Long bones continue to lengthen and thicken during infancy and

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youth. Interstitial growth of the epiphyseal plates. that are located between the diaphysis and epiphysis of the long bones. cause lengthening. The hyaline cartilage cells located in the epiphyseal plate are arranged in tall columns. The cells at the superior aspect divide rapidly causing the thickness of the plate to increase. This results in the epiphyses being pushed away from the diaphysis causing the bone to lengthen. The older chondrocytes enlarge and the surrounding matrix calcifies. These cells ultimately die and their matrix deteriorates leaving spicules just below the epiphyseal plate. Osteoblasts then cover the spicules forming cancellous bone that is eventually resorbed by the osteoclasts. resulting in an enlargement of the medullary cavity. This process of lengthening continues until the end of adolescence when the epiphyseal plates divide at a much slower rate causing the plate to become thinner. The epiphyseal plate ultimately is replaced by bone tissue and the epiphysis and diaphysis fuse.

1.1.6 Remodelling of Bone

Constituents of bone are continually being turned over by the concurrent processes of bone deposition and resorption so that bone is constantly being remodelled even after bone growth has stopped. During growth and development of the skeleton. deposition exceeds resorption leading to a positive skeletal balance. Once adult bone mass has peaked. usually within the early third of life. the skeleton is in equilibrium: deposition and resorption are equal. After a decade of zero balance. a slight excess of resorption over deposition with every remodelling cycle becomes evident. Over the subsequent years. the negative skeletal balance will become progressively greater correlating with the well-documented age-related decline in bone mass (Kleerekoper.

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1996). Remodelling of bone occurs to maximize the effectiveness of the skeleton in its mechanical uses and to maintain the plasma calcium levels (Sherwood. 1993).

1.1.7 Biochemical Markers of Bone Activity

Many biochemical markers for remodelling are present in bone that are important in providing information about bone loss and can be useful in determining therapies. They can also provide accessory information to bone density measurements. Resorption. or bone turnover. involves the removal of minerals from the skeleton by osteoclasts. These minerals then enter into the circulation and are subsequently cleared by the kidneys. Measurement of hydroxylprolines and pyridinolines in urine excretion are both markers of bone resorption. Hydroxylproline is the most abundant amino acid in collagen where pyridinolines cross-link the non-triple helical portions of one collagen molecule to another. An increase in excretion of either of these markers will translate to an increase in bone resorption (Kleerekoper. 1996). Bone formation occurs when the secretory products of osteoblasts fill in the resorption cavity from its base to the previous surface. As the cavity is filled in. mineralization begins from the base to the surface.

Measurement of alkaline phosphatase (ALP) is a marker for osteoblastic activity. ALP catalyzes the hydrolysis of phosphate esters at an alkaline pH (Harris et al. 1989). Gene expression characteristically begins immediately after the cessation of cell proliferation. reaching a maximum during matrix maturation and declining as matrix mineralization commences (Risteli et al.. 1993: Stein et al.. 1990). Total ALP activity has been recognized as a reliable indicator of osteoblast function. Although the greatest fraction of ALP in serum is from osteoblasts. contribution from hepatic. renal. and

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intestinal sources is significant, reducing the specificity and clinical use of ALP as a bone marker *in vivo*.

Osteocalcin (bone γ-carboxyglutamic acid) is another gene marker of the osteoblast and is the major non-collagenous protein of bone. Although the precise biological function of osteocalcin has not yet been fully elucidated, osteocalcin is known to be produced by mature osteoblasts during the mineralization phase and is only marginally detectable during the earlier phases of proliferation and matrix maturation (Millet et al.,). Osteocalcin has been suggested to act as either a cytokine or as a chemoattractant for osteoblasts, osteoclasts, and blood monocytes (Malone et al.,1982 : Price et al.,1985). This vitamin K dependent protein reflects both resorption and deposition. The serum level of osteocalcin has a component of both deposition and formation as it is incorporated in to the matrix during bone deposition and then released into the circulation during the resorptive phase (Kleerekoper, 1996).

1.2 MEDICAL RELEVANCE

1.2.1 Osteoporosis

Osteoporosis is a systemic skeletal disease that primarily affects elderly individuals. It is characterized by a significant decrease in bone mass and deterioration of bone tissue at a microarchitecural level (Consensus Development Conference, 1993). Affecting over 1.4 million Canadians, it is one of the most widespread chronic conditions facing the elderly (The Osteoporosis Society of Canada, 1999). One in four women and one in eight men will be affected with this disease that results in disfigurement, lowered self-esteem, reduction or loss of mobility, and decreased independence.

Age-related changes in estrogen secretion are one of the major reasons for the onset of postmenopausal osteoporosis in women (Turner et al., 1994). In men and women, maintenance of bone mass requires adequate levels of sex steroids (Vanderschueren et al., 1995). In women, reduced plasma levels of estrogen is assumed to either impair osteoblast-specific function directly or by paracrine mechanisms indirectly. This leads to an increase in relative osteoclastic resorption and uncoupling of the tightly regulated bone formation and resorption cycle with the net result being a loss in bone mass. (Heaney et al., 1978). The more prevalent problem of post-menopausal osteoporosis has been the focus of many clinical studies. However there have only been a few gender-specific studies exploring treatment options in men. Idiopathic osteoporosis in middle-aged men generally has a low bone turnover state, for which there are currently no available anabolic agents (Kurland et al., 2000).

The cause of osteoporosis is not fully elucidated but its multifactorial pathogenesis can be attributed to a combination of three elements: a reduction in absolute bone volume (osteopenia), repetitive strains resulting in microfractures leading to mechanical incompetence, and predisposing medical conditions (Frost, 1985). Medical conditions may include hormonal influences, calcium intake, race, skeletal size at maturation, genetics, cigarette and alcohol abuse, fitness, and chronic illness (McKenna et al., 1987, Osteoporosis Society of Canada, 1999). Hormones also influence bone remodelling. Important hormones that are being studied to combat this disease are estradiol, parathyroid hormone, and 1,25-dihydroxyviatmin D.

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1.2.2 Skeletal Dysplasias

The incidence of skeletal birth defects in the United States is approximately 1 in 130 live births (March of Dimes Perinatal Data Centre. 2000). These abnormalities of the skeletal system are due to inborn errors of bone growth and/or differentiation which occur in the developing fetus and affect the cartilaginous or bone-forming stage of bone development (Spranger. 1991). Two classifications of skeletal defects exist: dysplasias are those in which a tissue defect causes the abnormality, whereas dysostoses are defects of individual bones, either alone or in combination (Opitz et al., 1979). Although the individual bones are shaped abnormally in both cases, the dysostotic bone appears normal through biochemical and histological examination, whereas the dysplasic bone appears abnormal (Spranger. 1991).

Dysplasias are primarily characterized by a short-limbed dwarfism resulting from defective growth and their abnormalities are bilaterally symmetrical (Spranger. 1991). Achondroplasia, the most common form of short-limb dwarfism, occurs in approximately 1 in 26.000 to 1 in 40.000 births (Greenberg Centre for Skeletal Dysplasias. 2000). The main problem is the lack of ability to convert cartilage into bone. Although there is abnormal endochondral ossification, membranous ossification is not affected. Individuals with achondroplasia have disproportionate short stature with the final adult height range of 4 ft due to the shortening of the proximal ends of limbs. The fibroblast growth factor receptor 3 (FGFR3) is thought to be involved in this bone abnormality by altering the mitogenesis and/or differentiation due to constitutive activation of the receptor (Henderson et al., 2000).

1.3 HORMONAL FACTORS AFFECTING BONE

The development and maintenance of bone is affected by the interaction of various hormonal factors. Any deviation from the expected levels of hormones being delivered to the bony material may result in a change in the balance between formation and resorption. Estrogen, vitamin D, parathyroid hormone, and recently, the natriuretic factors, have been identified as key regulators of the skeletal system.

1.3.1 Estrogen

Three major types of estrogen are endogenous to the female body: estradiol, estrone, and estriol. Estradiol is the primary secretory product of the ovary whereas most estrone and estriol are formed in the liver from estradiol or in periperhal tissues from androstenedione and other androgens (Katzung, 1998). Estrogens act via two receptor subtypes found in the nucleus of the cell: estrogen receptor α (ERα), and estrogen receptor β (ERβ) (Vidal et al., 1999; Mosselman et al, 1996; Kuiper et al., 1996). The two subtypes have almost identical binding domains and similar affinities to estrogenic compounds (Mosselman et al., 1996; Tremble et al., 1997; Kuiper et al., 1997). The transcriptional activation mediated by the two subtypes may differ as the N- and C-terminal transactivating regions have different amino acid sequences (Paech et al., 1997). Once bound, the estrogen-receptor complex forms a homodimer that binds to a specific sequence of nucleotides called estrogen response elements (ERE) that are located on various genes. The genomic effects are mainly due to proteins synthesized in response to RNA transcribed by the gene. Furthermore, some genomic effects are indirect that are regulated by estrogen induced target cell proteins which are mediated by paracrine cytokine effects produced by neighbouring cells (Katzung, 1998). Combined with the

distribution of estrogen receptor subtypes in various tissues. the difference in the transcriptional regions could be important in defining estrogen response in the target tissue (Vidal et al.. 1999).

High affinity nuclear receptors are found on both osteoblastic and osteoclastic cell lineages. suggesting that estrogen can act directly on bone cells (Turner et al.. 1994; Erickson et al., 1988. Oursler et al. 1991). Its principal effect at the tissue level is to decrease bone resorption (Turner et al. 1994; Pacifici. 1996; Manolagas et al.. 1995). There is considerable evidence that much of this action is mediated by paracrine factors produced by osteoblasts which decrease osteoclast formation (Turner et al. 1994; Pacifici. 1996; Manolagas et al.. 1995; Jilka. 1998). ERα has been reported to be expressed in murine (Bellido et al.. 1993). rat (Davis et al.. 1994). and human osteosarcoma cell lines. as well as in cultured human osteoblast–like cells. and has also been detected by *in situ* PCR (polymerase chain reaction) in human osteoblasts. osteoclasts. and osteocytes. and by immunohistochemistry in human osteocytes (Ikegami et al.. 1994; Sutherland et al.. 1996; Ikegami et al.. 1993; Komm et al.. 1988). ERβ has been detected by semiquantitative RT-PCR (reverse transcription PCR) in rat osteoblasts and ROS 17/2.8 (rat osteosarcoma cell line). as well as in cancellous and compact bone from 8-week old rats (Onoe et al.. 1997). In humans. it has been detected in the osteoblast cell line SV-HFO also by RT-PCR (Arts et al.. 1997). ERβ is present in human osteosarcoma cell lines (MG-63 and SaOS-2) and in cultured human osteoblast-like cells. Immunoreactivity is seen in nuclei of murine and human osteoblast and osteocytes. and in the cytoplasm of osteoclasts (Vidal et al.. 1999).

1.3.2 Parathyroid Hormone (PTH) and Vitamin D

Parathyroid hormone (PTH) and vitamin D are principal regulators of calcium and phosphate homeostasis. PTH is a single chain peptide hormone that regulates calcium and phosphate flux across cellular membranes in bone and kidney, resulting in increased serum calcium and decreased serum phosphate (Katzung, 1998). It has been shown *in vitro* to stimulate bone resorption and inhibit bone formation (Raisz, 1993). Although PTH receptors have been found on mature and immature osteoblasts, they have not been identified on osteoclasts or osteocytes (Raisz, 1993). This suggests that the PTH-stimulated osteoblast may promote or permit osteoclastic activity. Although PTH enhances both bone resorption and formation, the net effect is to increase resorption. However, PTH in low doses my increase bone formation without first stimulating bone resorption (Katzung, 1998).

Vitamin D is a secosteroid produced in the skin from 7-dehydrocholesterol under the influence of ultraviolet radiation. It is also found in foods and is used to supplement dairy products. Vitamin D_3, or cholecalciferol, is the natural form, and the plant derived form is vitamin D_2, ergocalciferol (Katzung, 1998). The difference between these two forms is of little physiologic consequence in humans. It is a prohormone that is a precursor to many biologically active metabolites such as 1.25-dihydroxyvitamin D [1.25(OH)$_2$D] and 24.25-dihydroxyvitamin D [24.25(OH)$_2$D]. It is initially converted in the liver to 25-hydroxyvitamin D and then converted by the kidneys to the active metabolite. Vitamin D and its metabolites circulate in plasma tightly bound to a vitamin D-binding protein. Vitamin D increases calcium and phosphate resportion by 1.25(OH)$_2$D and bone formation may be increased by 24.25(OH)$_2$D. 1.25(OH)$_2$D is a

key regulator of both bone formation and resorption and influences the expression of genes related to the establishment and maintenance of the bone cell phenotype. Similar to PTH, the nuclear vitamin D receptors (VDR) are found on osteoblasts and not osteoclasts (Katzung. 1998). These receptors belong to the steroid/retinoid/thyroid hormone superfamily and act via binding to distinct vitamin D response elements (VDRE) (Yanaka et al.. 1998). Vitamin D down-regulates the expression of the type I collagen and bone sialoprotein genes but up-regulates osteopontin and osteonectin in humans and rats (Lichtler et al. 1989; Oldberg et al.. 1989; Owen et al. 1991).

Mediated through the osteoblast. vitamin D_3 has potent bone resorbing effects. In culture vitamin D inhibits cell proliferation (Owen et al.. 1991; Bonewald et al.. 1992), stimulates ALP activity (Farley et al.. 1994; Fedde. 1992). and increases osteocalcin production (Price et al.. 1980; Beresford et al.. 1984).

PTH is a potent stimulator of skeletal dynamics in men with idiopathic. low turnover osteoporosis. It is associated with substantial increases in lumbar spine and hip bone density and may prove to be an efficacious anabolic agent in men with osteoporosis (Kurland et al.. 2000).

1.3.3 Atrial Natriuretic Factors

Natriuretic peptides have been shown to play important roles in cardiovascular homeostasis, systemic blood pressure control and body fluid homeostasis (Matsukawa et al.. 1999; Burnett et al. 1984). They are also involved in the regulation of cardiovascular homeostasis by their potent natriuretic. diuretic. vasodilatory, and cell growth inhibitory activities (Suda et al.. 1998). Three isoforms currently exist: atrial natriuretic peptide

(ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (Matsukawa et al.. 1999; Yanaka et al.. 1998; Rosenzweig et al. 1991; Nakao et al.. 1992). ANP and BNP are expressed in large quantities in the cardiac atria and ventricles respectively (Maack. 1996, Ogawa et al. 1995, Mukoyama et al. 1991, Dagnino et al. 1991, Ogawa et al.. 1994), whereas CNP is most strongly expressed in the brain. but is also produced in vascular endothelial cells and in other tissues (Sudoh et al. 1990).

Biological functions of natriuretic peptides are mediated by particulate guanylyl cyclase linked membrane receptors: NPRA (GC-A) and NPRB (GC-B) (Schulz et al.. 1989, Maack et al. 1987, Chinkers et al.. 1991, Nakao et al.. 1992). These two receptors have cytoplasmic GC domains that are stimulated when the receptors bind ligand and increase intracellular cGMP (Yanaka et al.. 1998).

Ligand affinity for NPRA and NPRB is ANP $\geq$ BNP » CNP and CNP > ANP $\geq$ BNP respectively (Koller et al.. 1991, Suga et al.. 1992). ANP and BNP are thought to be endogenous ligands or NPRA. whereas CNP is selective for NPRB (Yasoda et al. 1998). NPRA is expressed in the vasculature. kidneys. and adrenal glands and its activation mediates vasorelaxant and natriuretic functions and decreases aldosterone synthesis. NPRB is expressed in the brain and may have a role in neuroendocrine regulation (Matsukawa et al.. 1999). However. the most widely and abundantly expressed receptor. NPRC. has a short intracytoplasmic extension with no GC activity. It is present in many but not all tissues that express a guanylyl cyclase receptor (Matsukawa et al.. 1999; Engel et al. 1994; Fuller et al.. 1988; Porter et al.. 1990). NPRC is thought to act as a clearance receptor and removes natriuretic peptides from the circulation (Maack et al.. 1987) and binds with the affinity order of ANP>CNP>BNP (Suga et al.. 1992). It

has been reported to control adenylate cyclase activity via G$_i$ protein, phospholipid hydrolysis, thymidine kinase activity, and mitogen-activated protein kinase activity in a variety of cells without any cGMP response. This suggests that it has physiological significance other than the clearance of ligands (Anand-Srivastava et al. 1990, Anand-Srivastava et al. 1996, Cahill et al. 1994, Hirata et al. 1996, Prins et al. 1996).

Recently, the natriuretic peptides have been shown to have a marked effect on the skeletal system. Transgenic mice with elevated plasma BNP levels in a study conducted by Suda et al. (1998) exhibited skeletal abnormalities, however no gross skeletal defects were observed at birth. Crooked tails were first observed 1-2 days post-partum and mice developed kyphosis, as early as 2-3 days after birth, becoming progressively hump-backed. Elongated limbs, paws, and tails developed, but no gross abnormalities were evident in the craniofacial portion. Increased body length was apparent in some animals in proportion to plasma BNP concentrations.

Matsukawa et al., (1999) established a knockout model for NPRC in mice. In this study, the -/- mice exhibited striking skeletal abnormalities recognisable 1 week after birth, similar to the mice over-expressing BNP. The abnormalities included hunched backs, dome shaped skulls, elongated tails, elongated femurs, tibias, metatarsal, and digital bones, longer vertebral bodies, increased body length, and decreased weight. The thoracic cages were also smaller and more constricted than the wild type. Development of secondary ossification centres in long bones was delayed in the -/- mice. In cartilage growth plates of 10-day-old -/- mice, cellular expansion was apparent in the zone of hypertrophic chondrocytes, but was no longer apparent in 3-month-old mice. There was no expansion evident in zones with resting or proliferating chondrocytes. The amino acid

compositions of organic bone matrix collagen were not different. Bony trabeculae in young -/- mice were thicker and longer than in the wild type. Strong expression of NPRC mRNA in osteoblastic cells lining the bony trabeculae of developing wild type bone was demonstrated, but NPRC mRNA was absent in the -/- mice. NPRB and CNP and the corresponding mRNAs have been demonstrated in cultured fetal mouse tibia (Yasoda et al., 1998) and together with this study suggest NPRC in growing bone modulates the autocrine/paracrine effects of locally produced natriuretic peptides, mainly CNP.

A recent study by Chusho et al. (2001) demonstrates that transgenic mice lacking the gene for CNP showed severe dwarfism as a result of impaired endochondral ossification. These mice exhibited characteristics similar to patients with achondroplasia. The length of both femurs and tibiae in the -/- mice were significantly shorter than those in the +/+ mice. Premature death also occurred among these mice.

These studies all indicate a potentially important role of the atrial natriuretic peptides, particularly CNP, in skeletal development. Determining the mechanism of action of CNP at a cellular level may provide valuable insight into combating disorders such as osteoporosis and skeletal dysplasias.

1.4 HYPOTHESIS

Studies on diseases such as osteoporosis and skeletal dysplasias have established contributing roles of certain hormones to the onset and progression of these conditions. Estrogen has already been shown in previous studies to be involved in bone pathophysiology, whereas the actions of atrial natriuretic factors are yet to be fully elucidated. A comparison of the effects between estrogen and CNP may provide useful

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information in determining how and where these hormones act. Cell culture has been a popular method of determining actions of hormones at a cellular level. Previous studies have shown that estradiol decreases cell proliferation in osteoblast-like cells but increases ALP activity. CNP has been shown to potentially increase ALP activity in some osteoblast-like cells, but another study showed CNP had a negligible effect. It is hypothesised that estradiol will have a greater effect than CNP on cell growth and alkaline phosphatase activities.

Organ culture of bones has been a technique used to elucidate the potential roles of these hormones in skeletal growth, remodelling, and maintenance. Previous studies have offered data, which indicate that the effect of estradiol in organ culture is negligible. Recent studies have shown, however, that CNP increased length of fetal bones in organ culture. It is therefore hypothesised that CNP, and not estradiol, is involved in skeletal growth during prenatal periods in the mouse.

The effects of CNP are hypothesised to have a greater effect on prenatal skeletal development than estradiol. This may lead to the confirmation of the importance of the natriuretic peptide system in skeletal development. Estradiol is expected to have a more profound effect on mature osteoblasts in culture, leading to the hypothesis that it plays a more important role in mature bone than in fetal bone development.

1.5 OBJECTIVES

The objectives of this study are to assess the effects of estradiol and CNP on bone development and growth using two different models: an *ex vivo* model of organ culture and an *in vitro* model of cell culture.

Chapter 2: The effects of estradiol and CNP on the cellular activity of the mature human osteoblastic cell line, SaOS-2

1. To develop cell culture techniques measuring cell proliferation and alkaline phosphatase activity (a measure of osteoblast function).

2. To assess the actions of estradiol and CNP using these techniques.

Chapter 3: The effects of estradiol and CNP on the growth and development of fetal mouse long bones.

1. To develop an organ culture system to characterise the impact of estradiol and CNP on bone growth *in vitro*.

2. To assess the effect of these hormones on the growth of long bones using this model system.

Chapter 2

The effects of estradiol and CNP on the cellular activity of the mature human osteoblastic cell line, SaOS-2

2.1 INTRODUCTION

Cell culture is a technique that has been used extensively in research in elucidating mechanisms at a cellular level. With the complex nature of bone, it is necessary to look at the individual responses of the cell types to various agents. Although whole bone explants are excellent models for the net outcome, the diverse cellular activities within the bone are not able to be studied. Using isolated bone cells, it may be easier to examine metabolic controls that are exerted ultimately at a cellular level. Cell culture may allow insight into the biochemical basis of bone formation and resorption.

2.1.1 SaOS-2 Cells

The SaOS-2 cell line, obtained from American Type Culture Collection (Rockville, MD), is a human tumour cell line isolated from the femur of an 11-year-old Caucasian female. This human osteosarcoma cell line is a prime model for cell culture as most malignant cell lines express differentiated features of the tissue origin along with cellular immortality (Sato, 1981). This makes it possible to study osteoblast-specific products and phenotype-related cellular functions. Set properties believed to be associated with the osteoblastic phenotype have been established from previous studies using isolated cells from embryonic calvaria, osteosarcoma, and other types of bone. These properties include elevated alkaline phosphatase, parathyroid hormone-stimulatable adenylate cyclase, synthesis and secretion of type I collagen, production of mineralised matrix (in Millipore filter chambers), and expression of bone γ-carboxyglutamic acid-containing protein, fibronectin, osteonectin, sialoprotein, proteoglycans, collagenases and others. Modulations of above properties by

1,25(OH)$_2$D$_3$ (Vitamin D), PTH, glucocorticoids, prostanoids and other growth factors are also important in establishing a cell line (Rodan et al., 1984). In a study by Rodan et al. (1987), these properties were established. The SaOS-2 cell line demonstrates an elevated level of alkaline phosphatase at confluence, stimulation of PTH-sensitive adenylate cyclase and expression of 1,25(OH)$_2$D$_3$ receptors which are found in fetal bone cells, mouse bone cells, osteosarcoma cells, and many others. SaOS-2 cells also form a calcifying matrix in diffusion chambers similar to bone marrow cells (Ashton et al., 1981), calvaria cells (Simmons et al., 1982), and osteoblastic rat osteosarcoma cell lines 17/2 and 17/2.8 (Shteyer et al., 1986). Osteonectin is present in SaOS-2 culture, however osteocalcin is not.

2.2 METHODOLOGY

2.2.1 Cell Line

SaOS-2 cells, obtained from the American Type Culture Collection (Rockville, MD), were taken from a cryotube stored in liquid nitrogen storage and rapidly thawed in a 37°C shaking water bath. Cells were then transferred into a 25 cm^2 culture flask with 5 ml of media (McCoy's 5A, 10% fetal calf serum, 0.1% gentomycin, and 1% HEPES buffer). Cells were kept in a humidified atmosphere of 5% CO$_2$ in air at 37°C incubator for two days to allow firm attachment. Media was changed every two days until confluence was reached. After reaching confluence, the cells were detached by treatment with 3 ml of 0.04% trypsin, washed with medium, and replated into a 75cm^2 flask at a density of 1 x 10^6 cells/15 ml.

2.2.2 Passaging of Cells

SaOS-2 cells were maintained in a 75 cm^2 plastic cell culture flask in culture medium (McCoy's 5A, 10% fetal calf serum, 0.1% gentomycin, and 1% HEPES buffer). The cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C incubator for two days to allow firm attachment. Media was changed every two days until confluence was reached. After reaching confluence, the cells were detached by treatment with 5 ml of 0.04% trypsin for 2 minutes at room temperature. Trypsin was then aspirated off and the flask was placed in 37°C incubator for 3 minutes. The flask was then removed from the incubator and 10 ml of medium containing 10% FCS was added to neutralize trypsinization. Cells were detached from the bottom of the flask with the media and cell solution was transferred into a 15 ml centrifuge tube. Cells were centrifuged for 5 minutes at 10000 g. After cells were pelleted, medium was aspirated off and the cells were resuspended in 10 ml of serum free medium. In order to establish cell concentration, 40 μl of cell solution was mixed with 40 μl of trypan blue solution and pipetted evenly into a haemocytometer. Live cells were counted using a Leitz inverted microscope at 10X magnification. An average of the four chambers in the haemocytometer was taken and used to calculate the total live cell count (TLCC). The TLCC was then used to calculate the volume of resuspended cells needed to plate the desired density (See Appendix A).

2.2.3 Phase Contrast Microscopy

In order to enhance the effects of estradiol and CNP, a reduced supplementation of fetal calf serum is employed. In order to determine viability of cells, a morphological

comparison of cultures supplemented with 10% FCS and 2%FCS was done. SaOS-2 cell cultures. under these conditions. were examined under a Leitz phase contrast microscope at 10X magnification to determine if there were any differences in cell growth or characteristics. Cells were photographed at 48. 72. 96. 144. and 168 hours after passaging using a Leitz Orthomatic camera microscope and colour slide film. Images were then scanned by Binuscan into JPEG format.

2.2.4 Preparation of Estradiol and C-Type Natriuretic Peptide (CNP)

Stock concentration of estradiol (generous donation from Dr. S.H. Shin from the Department of Physiology. Queen's University) was diluted in 100% ethanol to a final concentration of 10^{-2} M and stored at 4°C until use.

C-Type Natriuretic Peptide was obtained from Sigma chemical company (Oakville. ON). It was diluted in 0.01 M acetic acid to a final concentration of 10^{-4} M. The solution was aliquoted into 100 µl samples and stored at -70°C.

2.2.5 Cell Proliferation

^{3}H-Thymidine Incorporation

Cells were passaged into 24 well plates at a concentration of 1×10^4 cells/ml in 1.5 ml of media (McCoy's. 2% FCS. 0.1% penicillin & streptomycin. and 1% HEPES buffer). Cells were allowed to firmly attach for 48 hours in an air incubator. Normal growth media was replaced with synchronization media (McCoy's. 1% CS. 0.1% gentomycin. 1% HEPES buffer) and cells were cultured for 24 hours.

Following synchronization, cells were supplemented with one of three compositions of media. The control group were supplemented with 2% FCS, the maximal group with 15% FCS and the negative group were serum free. Radiolabelled ^{3}H-thymidine at 0.5 μCi/ml was added to each plate for 48 hours.

Effects of Estradiol

Twenty-four hours after synchronization, original media composition was introduced and concentrations of estradiol between 10^{-18} and 10^{-9}M were administered with 0.5 μCi/ml ^{3}H-thymidine.

Effects of CNP

Original media composition was introduced following synchronization and concentrations of CNP between 10^{-10} and 10^{-7}M were administered with 0.5 μCi/ml ^{3}H-thymidine.

Detection of Thymidine Incorporation

Thymidine incorporation was terminated after 48 hours of cell growth. This was accomplished by the aspiration of the media. Cells were washed with McCoy's to remove excess ^{3}H-thymidine and then fixed with ethanol:acetic acid (3:1) for 10 minutes. Fixative was removed and cells were washed with water. Acid insoluble material was precipitated by incubation with cold 0.5 M perchloric acid (PCA) to remove all unincorporated ^{3}H-thymidine. DNA extracted into fresh PCA by heating to 80 C for 20 minutes. Solution was then transferred from each plate into separate scintillation vials containing 10 ml Beckman Ready Value Liquid Scintillation Cocktail. Incorporated radioactivity was determined in a liquid scintillation spectrometer.

<u>Colorimetric Assay</u>

The cell proliferation assay. using CellTiter 96® Aqueous One Solution. (Promega; Madison. WI) is a colorimetric method for determining the number of viable cells in proliferation. The solution contains a tetrazolium compound. MTS [3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent. PES (phenazine ethosulfate). The MTS compound is bioreduced by cells into a coloured formazan product by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge et al.. 1993). The coloured formazan product is read at an absorbance of 490 nm. Using a Spectramax 250 UV-VIS plate reader.

To test the viability of the study. cells were passaged in a 96 well plate at 1500 cells/well into 200 µl of media supplemented with either 2% FCS (control). 15% FCS (maximal) or serum free (negative) for 60 hours.

To determine the optimal concentration of cells to be plated for the experiments. various concentrations of cells were plated. SaOS-2 cells were passaged in a 96 well plate at concentrations between 300 and 3600 cells/well into 200 µl media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer) for 60 hours.

The optimal length of the experiments was then determined. Cells were passaged in a 96 well plate at 1500 cells/well into 200 µl of media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer). One column of cells was assayed every twelve hours to determine optimal length of culture.

Effect of Estradiol

Cells were passaged in a 96 well plate at 1500 cells/well into 200 µl of media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer) and administered doses of estradiol between 10^{-17} and 10^{-6}M.

Effects of CNP

Cells were passaged in a 96 well plate at 1500 cells/well into 200 µl of media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer) and administered doses of CNP between 10^{-16} and 10^{-6}M.

The cells treated with both estradiol and CNP were subject to the proliferation assay at the end of the 60 hour incubation. The CellTiter 96® Aqueous One Solution Reagent was thawed in a water bath at 37°C. The reagent was then pipetted into each well as 40 µl aliquots. The plate was then incubated for 3 hours at 37°C in a humidified 5% CO_2 atmosphere. Following incubation. the plate was read by the Spectramax 250 UV-VD plate reader at 490 nm.

2.2.6 Alkaline Phosphatase Activity

Cells were passaged at concentrations of 2×10^5. 1×10^5. 5×10^4. and 2.5×10^4 cells/ml in 5 ml of media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer). Media was changed every 48 hours until cells reached confluence at day 6.

Effect of Estradiol

Cells were passaged into 60 mm^2 plastic dishes at a concentration of 100 000 cells/ml in 5 ml media (McCoy's. 2% FCS. 0.1% gentomycin. and 1% HEPES buffer)

and administered doses of estradiol lat 10^{-15}, 10^{-13}, 10^{-11}, and 10^{-9}M. Media was changed and estradiol reintroduced every 48 hours until cells reached confluence at day 6.

Effects of CNP

Cells were passaged into 60 mm^2 plastic dishes at a concentration of 100 000 cells/ml in 5 ml media (McCoy's, 2% FCS, 0.1% gentomycin, and 1% HEPES buffer) and administered doses of CNP at 10^{-11}, 10^{-9}, and 10^{-7}M. Media was changed and CNP reintroduced every 48 hours until cells reached confluence at day 6.

Following incubation, cells were scraped using 1X PBS solution and pelleted by centrifugation at 10.000g for 5 minutes and supernatant was aspirated. Cell pellets were then homogenized for 1 minute with 0.5 ml of distilled water. Following homogenization, an additional 0.5 ml of distilled water was added to dilute the cell solution. Solutions ranging from 0 nM to 1000 nM of p-Nitrophenol standard (Sigma: St. Louis, MO) were made and used as standards for the experiment. In 7 ml culture tubes kept on ice, 100µl of sample (or standard) were added to 400 µl of equal volumes of 0.1M glycine-NaOH buffers, 0.1% Triton X 100/Saline (0.9%NaCl), distilled water, and 15 mM para-nitrophenol-phosphate, pNPP (or distilled water for samples).

Tubes were incubated for 5 minutes at room temperature. To stop the reaction 125 µl of 2M NaOH was added to each tube and placed on ice. A 300 µl aliquot of each sample was transferred to a 96 well plate and read on a Spectramax 250 UV-VIS plate reader at 405 nm.

2.2.7 Statistical Analysis

Statistical analysis was done by a one-way analysis of variance followed by a Student-Neuman-Keul's test for multiple comparison (Sigma Stat 2.0: Jandel Corporation. 1995). Data are expressed as mean ± standard deviation. unless otherwise indicated. A p-value less than 0.05 is considered to be statistically significant.

2.3 RESULTS

2.3.1 Phase Contrast Microscopy

Cultured cells in media supplemented with 10% and 2% FCS were examined under phase contrast microscopy and no difference in cell growth or characteristics is evident (Figure 2-1). Confluence is similar at all time points between the two supplement conditions. Cells in both conditions are large and irregularly shaped with abundant cytoplasm and a prominent nucleus.

2.3.2 Cell Proliferation

^{3}H-Thymidine Incorporation

In determining the viability of the assay. media supplementation was found to affect the incorporation of ^{3}H-thymidine (Figure 2-2). Cells grown in a serum free environment had a 45% decrease in incorporation from control conditions (2% FCS) whereas an increase to 15% FCS caused an increase in incorporation of 20 %.

Estradiol at concentrations of 10^{-13} and 10^{-9} M caused an increase of ^{3}H-thymidine uptake 36 % and 33 % respectively from control conditions (Figure 2-3). No other doses of estradiol caused a significant change from control conditions.

CNP had no effect on ^{3}H-thymidine incorporation into the cells (Figure 2-4). This indicates that there was not an increase in cell division occurring in the cell from control.

Colorimetric Assay

Cells grown in a serum free environment had a 52% decrease in cell proliferation (Figure 2-5). However. there was no significant change between control conditions (2% FCS) and maximal conditions (15% FCS).

33

10 % FCS   2 % FCS

48 hours
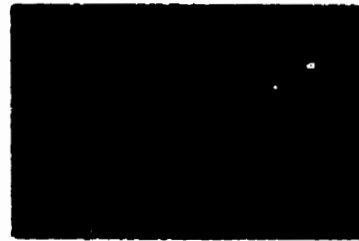
72 hours

96 hours

144 hours

168 hours

Figure 2-1
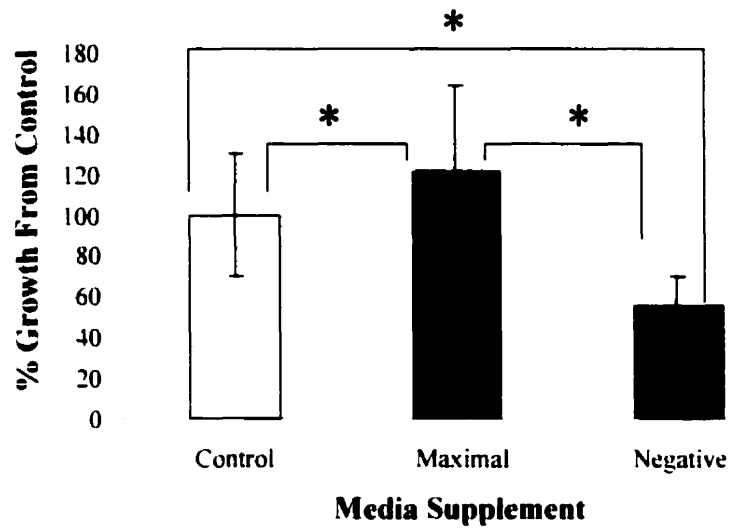
Figure 2-2: DNA synthesis determined by amount of ^{3}H-thymidine incorporated into cells. Control denotes cells supplemented with 2% FCS (n=13). maximal denotes cells supplemented with 15% FCS (n=13) and negative denotes cells in a serum free environment (n=13). There is a significant difference in per cent growth between the control. maximal. and negative groups. Values are mean ± standard error and calculated as per cent of control. * indicates significance at p<0.05.

Concentration of Estradiol (log X) (M)
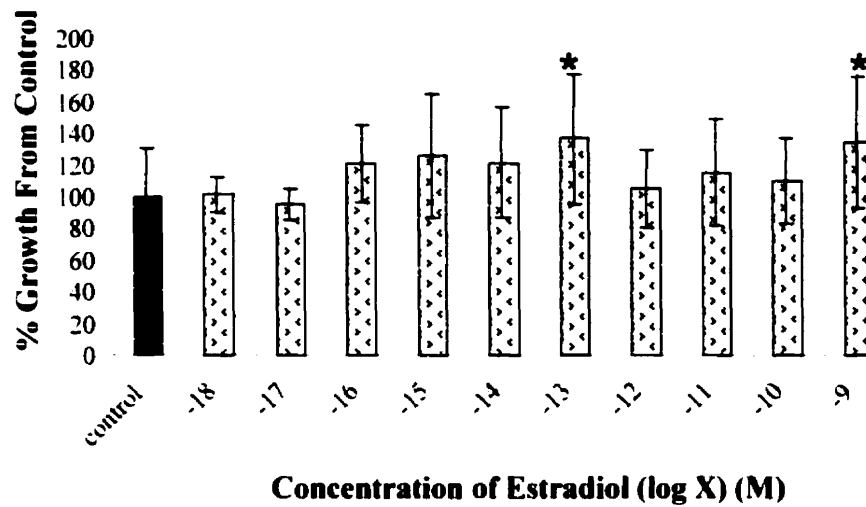
Figure 2-3: Cell proliferation determined by amount of ^{3}H-thymidine incorporated into cells. Cells cultured with 2% FCS and with a dose of estradiol at concentrations of 10^{-18} (n=3), 10^{-17} (n=3), 10^{-16} (n=7), 10^{-15} (n=13), 10^{-14} (n=13), 10^{-13} (n=13), 10^{-12} (n=13), 10^{-11} (n=12), 10^{-10} (n=10), and 10^{-9} M (n=9) are compared to control. Control denotes cells supplemented with 2% FCS (n=13). There is a significant increase at 10^{-13} and 10^{-9} M concentrations from control. All other concentrations are not significantly different. Values are mean ± standard error and calculated as per cent of control. * indicates significance at p<0.05 from control.

Concentration of CNP (log X) (M)
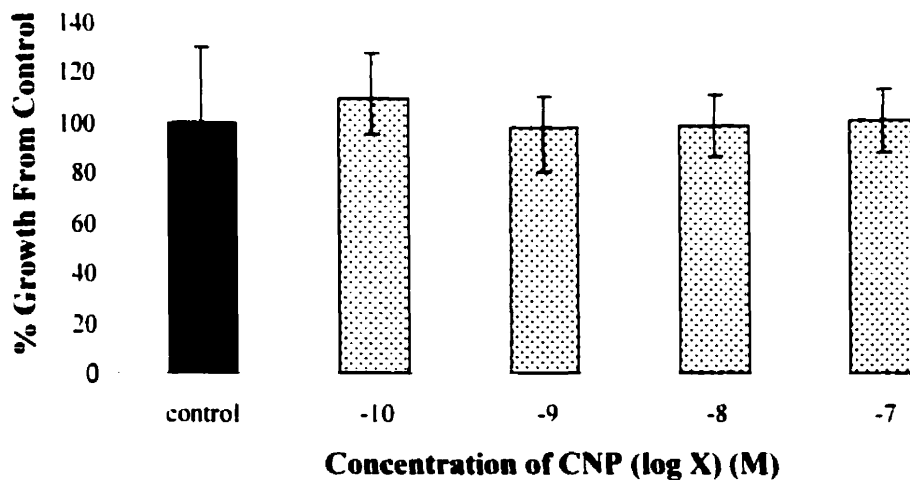
Figure 2-4: Cell proliferation determined by amount of ^{3}H-thymidine incorporated into cells. Cells cultured with 2% FCS and with a dose of CNP at concentrations of 10^{-10} (n=2), 10^{-9} (n=5), 10^{-8} (n=5), and 10^{-7} M (n=3) are compared to control. Control denotes cells supplemented with 2% FCS (n=13). There is no significant difference between control and the cells treated with CNP. Values are mean ± standard error and calculated as per cent of control.
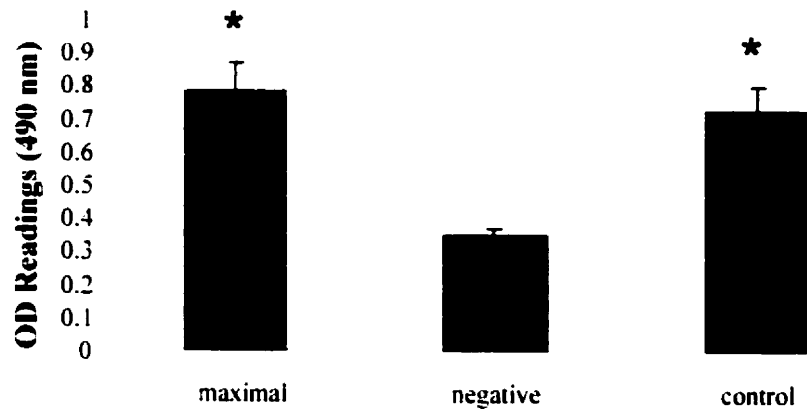
Figure 2-5
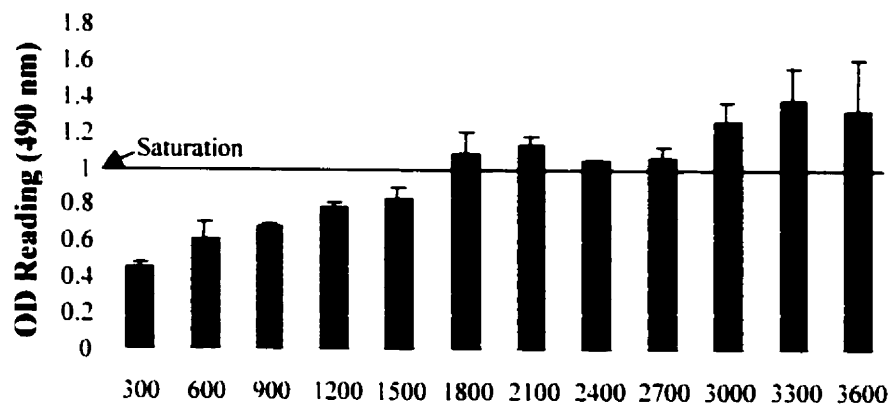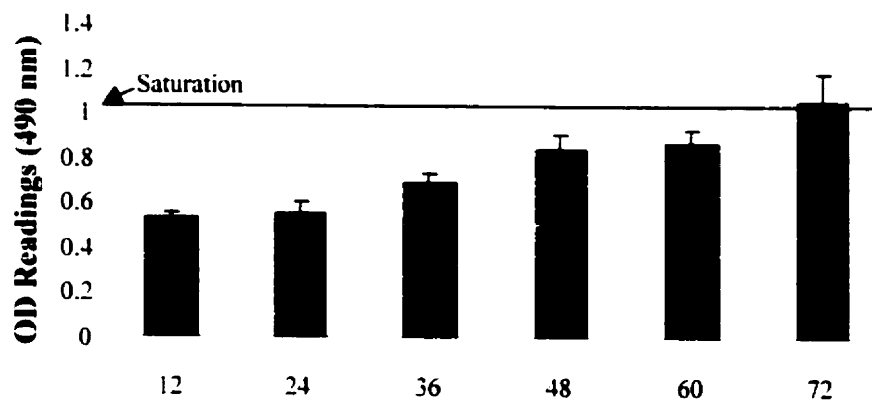
Figure 2-6

Figure 2-7

In order to determine the optimal concentration of cells to be plated, saturation of the solution must occur. This happens when OD readings at 490 nm reach 1.0. After 60 hours in culture, a plating concentration of 1500 cells/well approached this level (Figure 2-6). This was determined to be the highest concentration of cells that could be used for the experiment.

Optimal duration of incubation was then determined. The solution reached saturation at 72 hours in culture (Figure 2-7). To achieve optimal readings, the solution should not reach saturation. Therefore the optimal duration for culture is 60 hours from passaging.

Estradiol had no significant effect on cell proliferation (Figure 2-8). This is not in agreement with the previous experiment using ^{3}H-thymidine incorporation and indicates that metabolically active cells may not be active in cell division.

CNP had a no significant influence on cell proliferation from control (Figure 2-9). This concurs with the results from the ^{3}H-thymidine incorporation study. There is no effect of CNP on cell division or metabolic activity.

2.3.3 Alkaline Phosphatase

The standard curve for alkaline phosphatase has shown to be a linear fit read at 405 nm (Figure 2-10). All experiments were based on a linear fit curve.

In order to determine the effectiveness of the assay, different cell concentrations were plated as an increase in cell number will result in increased ALP activity. Alkaline phosphatase activity had a cell concentration dependent decrease between 2 x 10^5 and 2.5 x 10^4 cells/ml (Figure 2-11).

Concentration of Estradiol (log X) (M)
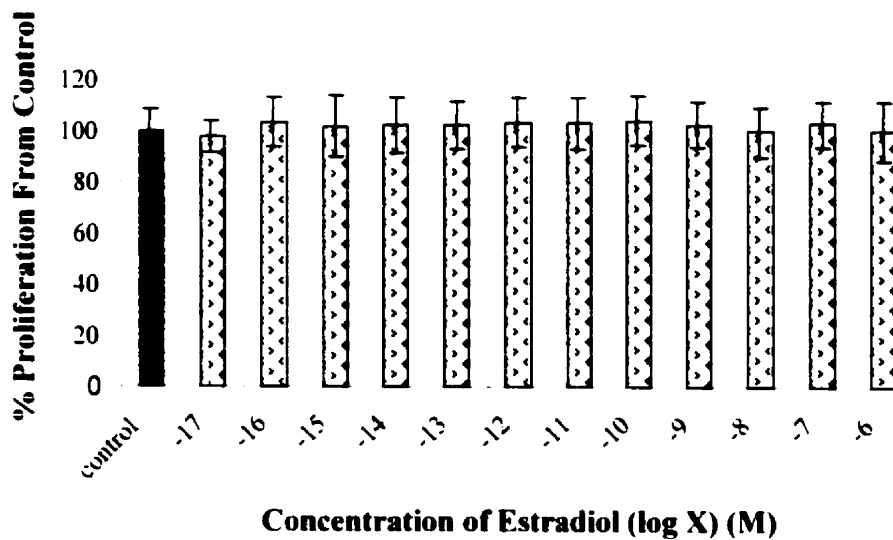
Figure 2-8: Proliferation in cells cultured with 2% FCS and treated with estradiol at concentrations of 10^{-17} (n=7), 10^{-16} (n=22), 10^{-15} (n=22), 10^{-14} (n=22), 10^{-13} (n=22), 10^{-12} (n=22), 10^{-11} (n=22), 10^{-10} (n=22), 10^{-9} (n=22), 10^{-8} (n=22), 10^{-7} (n=22), and 10^{-6} M (n=15). There was no significant difference among groups. Values are mean ± standard deviation and calculated as per cent from control (n=21) .

Concentration of CNP (log X) (M)
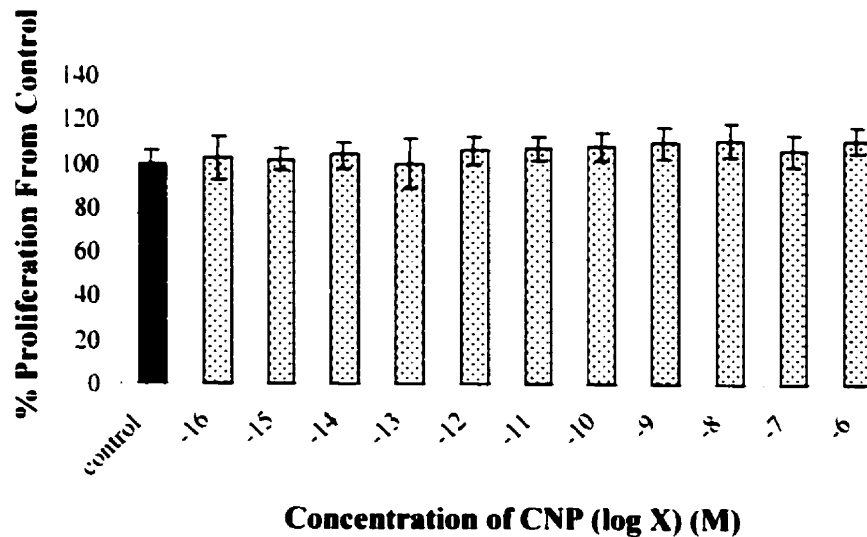
Figure 2-9: Proliferation in cells cultured with 2% FCS and treated with CNP at concentrations of 10^{-16} (n=12), 10^{-15} (n=10), 10^{-14} (n=14), 10^{-13} (n=14), 10^{-12} (n=14), 10^{-11} (n=15), 10^{-10} (n=13), 10^{-9} (n=14), 10^{-8} (n=12), 10^{-7} (n=14), and 10^{-6} M (n=15). There was no significant difference among groups. Values are mean ± standard deviation and are calculated as per cent from control (n=19)
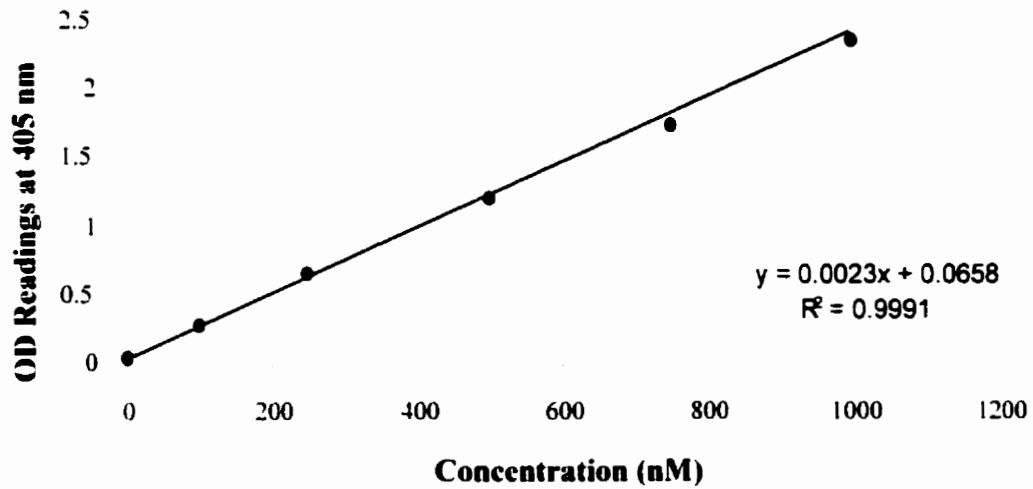
Figure 2-10: Standard curve for alkaline phosphatase activity.
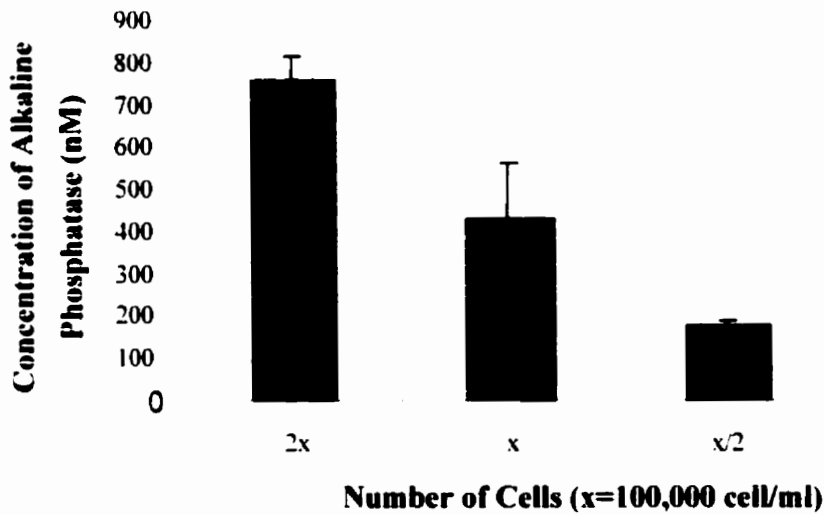
Figure 2-11: Alkaline phosphatase activity in varying concentrations of cells. Cells are cultured with 2% FCS (n=4). Values are mean ± standard deviation. There is a significant difference (p<0.05) between all concentrations.

Estradiol caused a significant increase in alkaline phosphatase activity at 10^{-15} M and a significant decrease at 10^{-9} M. No significant changes were observed at 10^{-13} or 10^{-11} M concentrations (Figure 2-12).

Alkaline phosphatase activity was significantly increased with cells cultured with 10^{-11} M of CNP. Effects of CNP at 10^{-9} and 10^{-7} M were negligible (Figure 2-13).
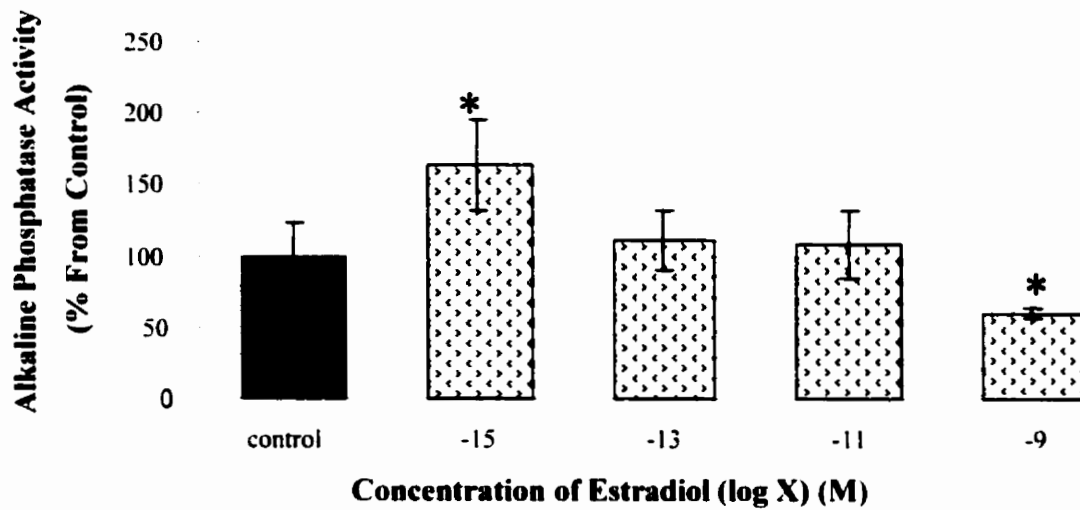
Figure 2-12: Alkaline phosphatase activity in cells cultured with 2% FCS and treated with estradiol at concentrations of 10^{-15} (n=10). 10^{-13} (n=16). 10^{-11} (n=10). 10^{-9} M (n=2). Values are mean ± standard deviation. There is a significant increase in ALP activity at 10^{-15} M and a significant decrease in ALP activity at 10^{-9} M compared to control. ✸ indicates significance at $p<0.05$ when compared to control.
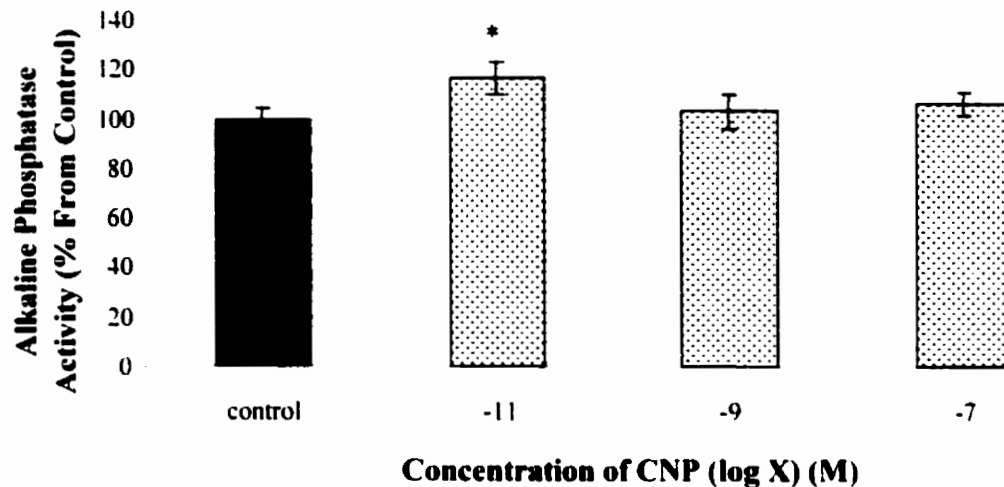
Figure 2-13: Alkaline phosphatase activity in cells cultured with 2% FCS and treated with CNP at concentrations of 10^{-11} (n=10). 10^{-9} (n=2). and 10^{-7} M (n=10). Values are mean ± standard deviation. There is a significant increase in ALP activity at 10^{-11} M when compared to control. * indicates significance at $p<0.05$ when compared to control.

Chapter 3

The effects of estradiol and CNP on the growth and development of fetal mouse long bones

3.1 INTRODUCTION

Bone is a very complex organ and the study of its physiology, pathology, and development is an active and exciting field. Furthermore, the interactions of the heterogeneous cell population and the bony matrix have been difficult to analyse. In order to determine the net effects of the cellular activities, an intact bone can be used in culture. Organ culture was initially documented by Wilhelm Roux and Leo Loeb when they described the maintenance of isolated embryonic fragments and developing organs *in vitro* in the 1800s (Stern et al., 1979). Although other organs were used in the early 1900's, it was not applied to bone tissue until Strangeways and Fell (1926) first demonstrated that the mesenchymal limb bud rudiment from a 3-day-old chick could survive *in vitro*. The limb bud differentiated into hyaline cartilage which was enveloped by a fibrous perichondrium. They also found that six-day femurs, after 21 days in culture, were able to grow and differentiate into a more mature bone from cartilaginous rod structures. Organ cultures have been able to provide valuable information about physiology, pathology, and bone development (Stern et al., 1979). Organ culture with fetal mice long bones has been shown to be a suitable model (Soskolne, 1986). The assay is described to be suitable for the study of bone modelling, measuring bone growth, formation, calcification, and resorption.

3.1.1 Embryonic Development of the Mouse

The female mouse copulates usually when ova are ready for fertilisation in estrus. Successful mating with a male mouse is indicated by the presence of a vaginal plug. The plug that occludes the vaginal orifice is a coagulum of fluid from the vesicular and

coagulating glands of the male (Rugh. 1968). Implantation of the embryo in the uterus occurs between day 4 and 5. The gestational period is usually between 18 and 21 days depending on the strain of mouse. The BJ6 strain has a gestational period of 21 days and the litter size usually averages between 10 and 11 mice (Rugh. 1968). Cartilage and fibrous membranes begin to form and ossify at about 12 days and by day 16. all skeletal parts are chondrified. In the femur and tibia. chondrification begins at about day 13 and the ossification centres appear around day 14. Ossification is not complete until birth.

3.2 METHODOLOGY

3.2.1 Animals

BJ6 mice were obtained from Charles River Laboratories and bred on the premises. Female mice were group housed under conditions of a 12-hour light/dark cycle. with room temperature at 22-24°C. Food and water were provided *ad libitum*. Male mice were isolated except when mating and were subject to the same conditions. Potential pregnancy was determined by presence of a vaginal plug in the morning following mating. The pregnant mice were isolated from the rest of the mice and were carefully observed until gestational day 17. Mice which did not exhibit pregnancy were placed back in group housing with other female mice and subsequently mated.

3.2.2 Surgical Procedures

On GD 17. pregnant females were given a lethal dose intraperitoneally of Somnotol (45mg/kg b.w.). Abdominal dissection was done in order to expose the abdominopelvic cavity. Embryos were carefully removed from each of the two uterine

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horns and placed in a petri dish with 10 ml Minimal Essential Media supplemented with 0.1% Gentomycin. Under a dissecting microscope, the amniotic sac was removed from the embryos and lower limbs were subsequently disembodied. The femurs and tibiae were left intact and dissected free of muscle and soft tissue.

3.2.3 Organ Culture

The femurs and tibiae were cultured for 8 days in 2 ml Minimal Essential Media (with Earle's Salts and L-glutamine. GIBCO) supplemented with 2 g/L BSA. 200 mg/L β-glycerol phosphate. 50 mg/L ascorbic acid. and 3% 100X penicillin/streptomycin in 35 mm plastic petri dishes. Culture dishes were place in an incubator with 5% CO_2 in air at 37°C.

Treatment With Estradiol and CNP

Concentrations of estradiol at 10^{-13}. 10^{-11}. 10^{-9}. and 10^{-7} M were added on day 0 in separate dishes. Media was replaced on day 4 and fresh estradiol was added. The experiment terminated on day 8.

Similarly. concentrations of CNP at 10^{-11}. 10^{-9}. and 10^{-7}M were added on day 0 in separate dishes. Media was also replaced with fresh CNP on day 4 and the experiment was terminated on day 8.

3.2.4 Photomicrographs and Measurements

Photomicrographs were taken of fetal mouse femurs and tibiae using an inverted microscope on day 0. day 4. and day 8 at 2.5X magnification using a Leitz Orthomatic camera microscope and Fuji colour slide film (100 ASA). All measurements were taken

from the photomicrographs using the Bioquant system. The maximal longitudinal length was measured as the total length. the maximal proximal and distal cartilaginous ends were measured as proximal and distal length. and the osteogenic centre was also determined by measuring the darker zone of the diaphysis (Figure 3-1). The total areas as well as proximal. distal. and osteogenic areas were also similarly measured.

3.2.5 Fixation and Embedding of Mouse Femur and Tibia

Bones were fixed by immersion in 4% paraformaldehyde in PBS fixative and stored at 4 C for three to four days. They were then dehydrated through 75%. 80%. and 90% ethanol solutions. Bones were then embedded using the LKB HISTORESIN Embedding Kit (LKB. Sweden) according to manufacturer protocol without decalcification. Briefly. bones were placed in a 1:1 dilution of 95% ethanol and infiltration solution for 1 hour. Specimens were then put in 100% infiltration solution for 2 hours. Following infiltration. each bone was placed in the lid of an electron microscopy molding capsule and embedding medium was added. The remaining portion of the capsule. with the end cut off. was placed on top of the lid and additional embedding solution was added until the entire capsule was full. Capsules were allowed to polymerize at room temperature for 2 hours and then placed in a 60°C oven overnight. Sections were cut using an ultramicrotome at 4μm.
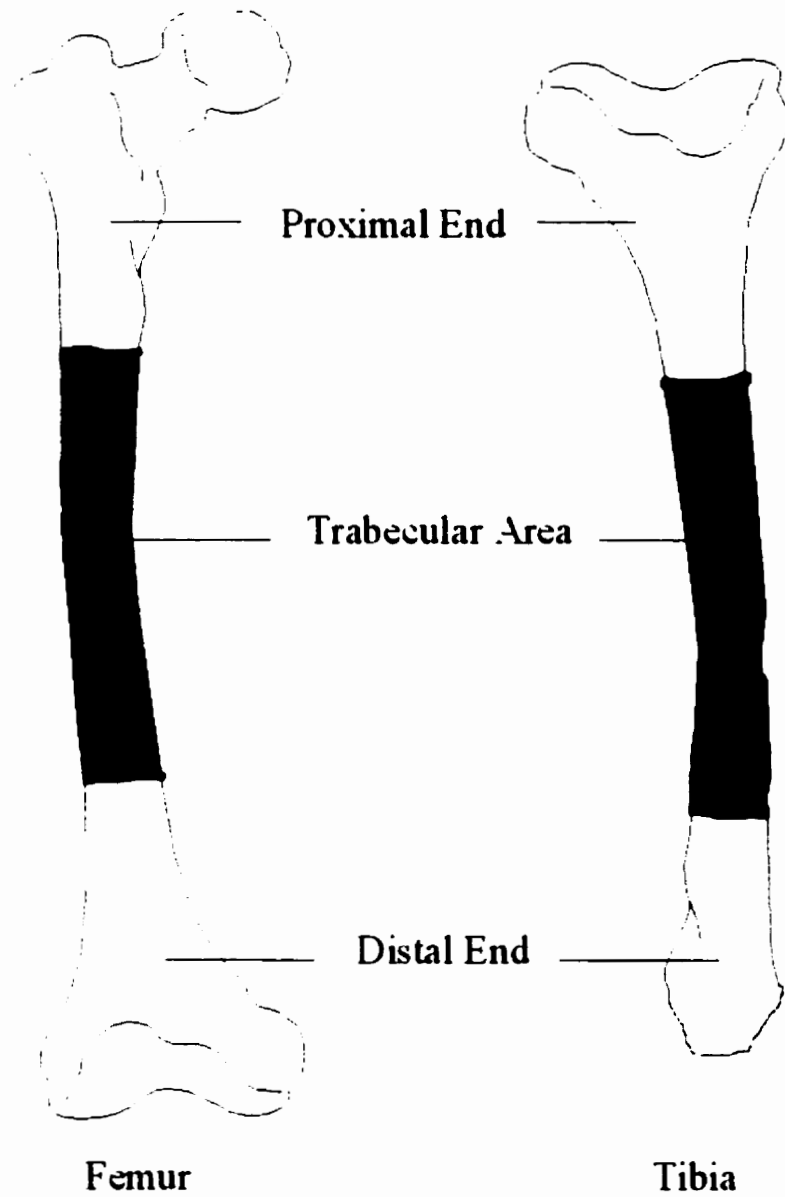
Figure 3-1: The length and area of the cartilaginous ends (light zone) and osteogenic centre (dark zone) were measured using the Bioquant™ system.

3.2.6 Morphological Studies

Toluidene Blue Staining

Femoral sections were hydrated through 100%, 95%, 80%, and 75%, ethanol solutions and rinsed in distilled water. They were then placed in 20% Methanol for 60 minutes and once again rinsed in distilled water. Sections were incubated at room temperature in a filtered solution of toluidene blue (1% toluidene blue, 1% sodium tetraborate) for 4 minutes. Sections were then dehydrated through 75%, 80%, 95%, and 100% ethanol solutions and into toluene. Dehydrated sections were then cover-slipped with Pro-Texx Mounting Medium (Fisher Scientific; Nepean, ON).

Measurement of Proliferative and Hypertrophic Zones

Examination of the toluidene blue stained femurs were done on the Bioquant system using a Leitz microscope at 4X magnification. Measurements were taken as length of proliferative or hypertrophic zones of total lengths in both the proximal and distal areas. The epiphyseal cartilage areas, both proximal and distal, are divided into zones starting from the outside. The resting zone consists of hyaline cartliage that does not have any morphological changes in the cells. The proliferative zone contains rapidly dividing chondrocytes which forms columns of stacked cells parallel to the long axis of the bone. The hypertrophic zone contains large chondrocytes with glycogen that has been accumulated in the cytoplasm giving the cell a bloated look.

Morphological Analysis

Sections were examined at 40X magnification using a Leitz microscope for any morphological differences. Areas examined were the perichondrium, resting zone, proliferative zone, hypertrophic zone, and trabeculae.

3.2.7 Statistical Analysis

Statistical analysis was done by a one-way analysis of variance followed by a Student-Neuman-Keul's test for multiple comparison (Sigma Stat; Jandel Corporation. 1995). Data are expressed as mean ± standard deviation. A p-value less than 0.05 is considered to be statistically significant.

3.3 Results

3.3.1 Establishment of Organ Culture Model

Initial studies were conducted to evaluate the success of the model in both embryonic mouse tibiae and femurs without any addition of drug. In both femurs and tibiae, a significant increase in growth was demonstrated in both length and area in proximal, distal, and total bone measurements (Figure 3-2 and 3-3). An increase in both trabecular area and length however was not seen.

3.3.2 Effect of Estradiol on Bone Growth

Photomicrographs of fetal mice tibiae taken at day 0, day 4 and day 8 were used to measure lengths and areas (Figure 3-4). There were no significant effects of estradiol on the culture of tibiae except at 10^{-13} M where an increase in total length and area was observed at day 4 (Figures 3-5 – 3-10). A significant increase in trabecular area in tibiae at day 8 was also observed.

Fetal mice femurs were also observed by photomicrographs at day 0, day 4, and day 8 (Figure 3-11). An increase in total area at day 4 of bones cultured with estradiol at 10^{-13} M was the only measurement reaching significance. All other parameters at all concentrations failed to reach significance (Figure 3-12 – 3-17).

3.3.3 Effect of CNP on Bone Growth

Photomicrographs of tibiae taken at day 0, day 4 and day 8 (Figure 3-18) were used to measure lengths and areas. No statistical significance was seen in proximal,

51
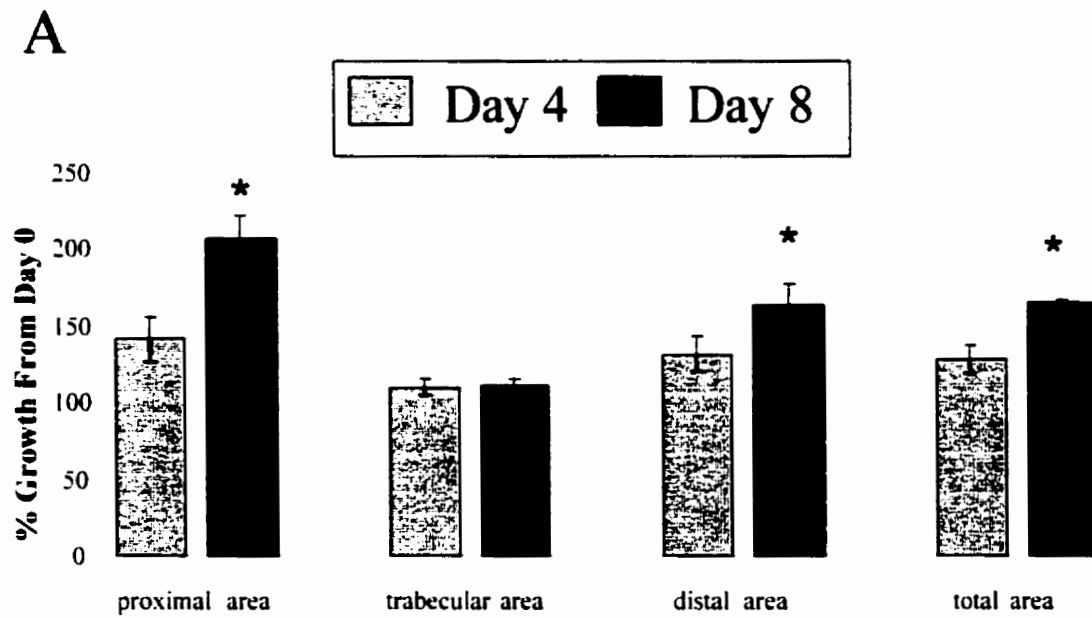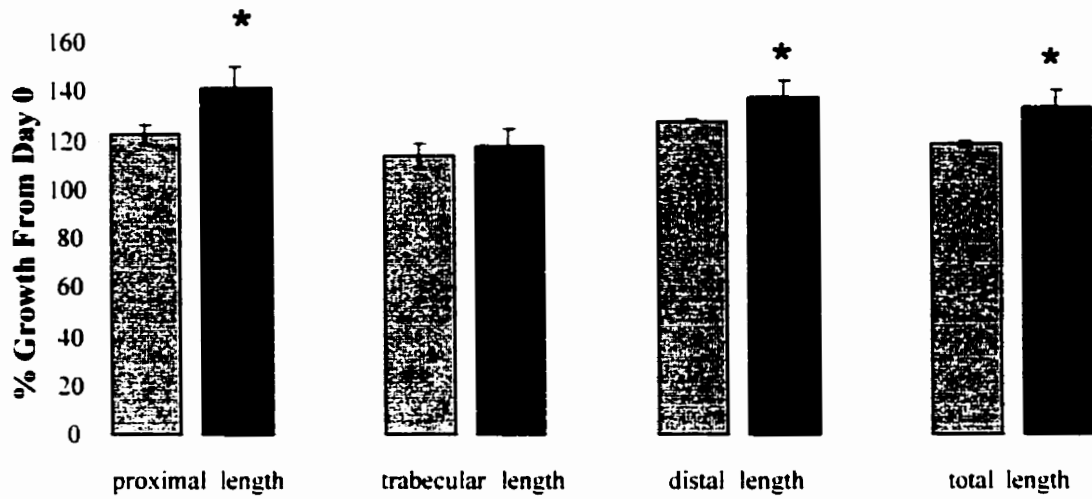
Figure 3-2: Growth of tibiae in organ culture. Lengths (A) and areas (B) were measured and calculated as percentage growth from day 0 on day 4 and day 8. There is a significant difference in both length and area between day 4 and day 8 in proximal, distal, and total lengths/areas, but not in trabecular values. Values are mean ± standard deviation. * indicates statistical significance from day 4. $p < 0.05$.
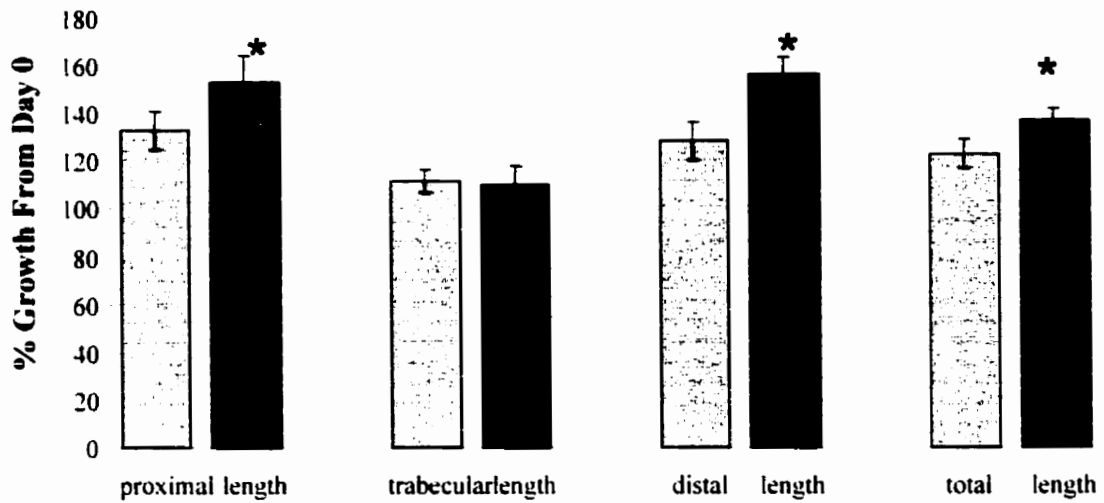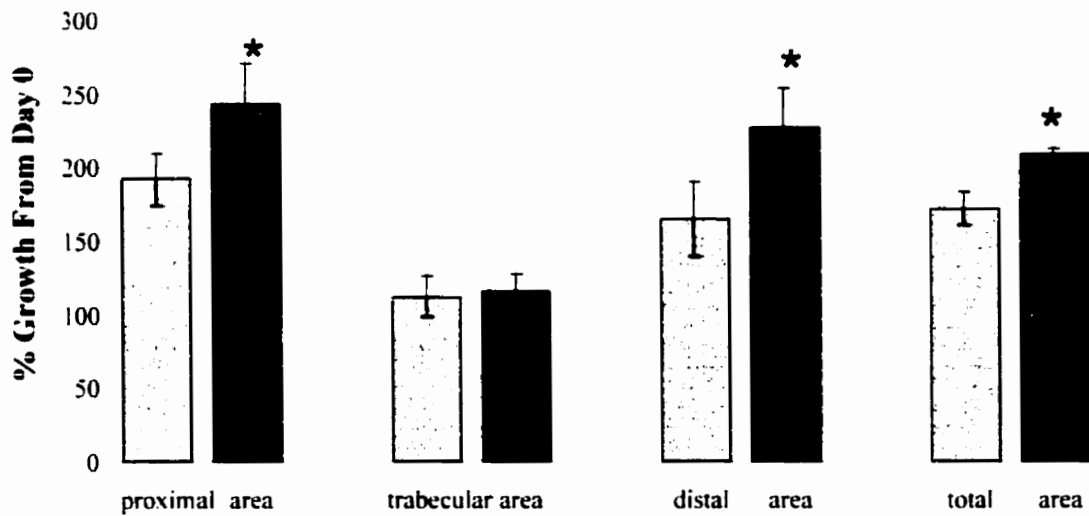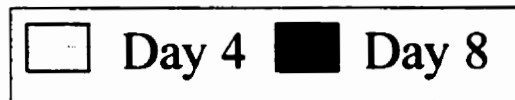
Figure 3-3: Growth of femurs in organ culture. Lengths (A) and areas (B) were measured and calculated as percentage growth from day 0 on day 4 and day 8. Values are mean ± standard deviation. There is a significant difference in both length and area between day 4 and day 8 in proximal, distal, and total lengths/areas, but not in trabecular values. * indicates statistical significance from day 4. $p < 0.05$.

	Control	Estrogen 10^{-13}	Estrogen 10^{-11}	Estrogen 10^{-9}	Estrogen 10^{-7}
Day 0					
Day 4					
Day 8					
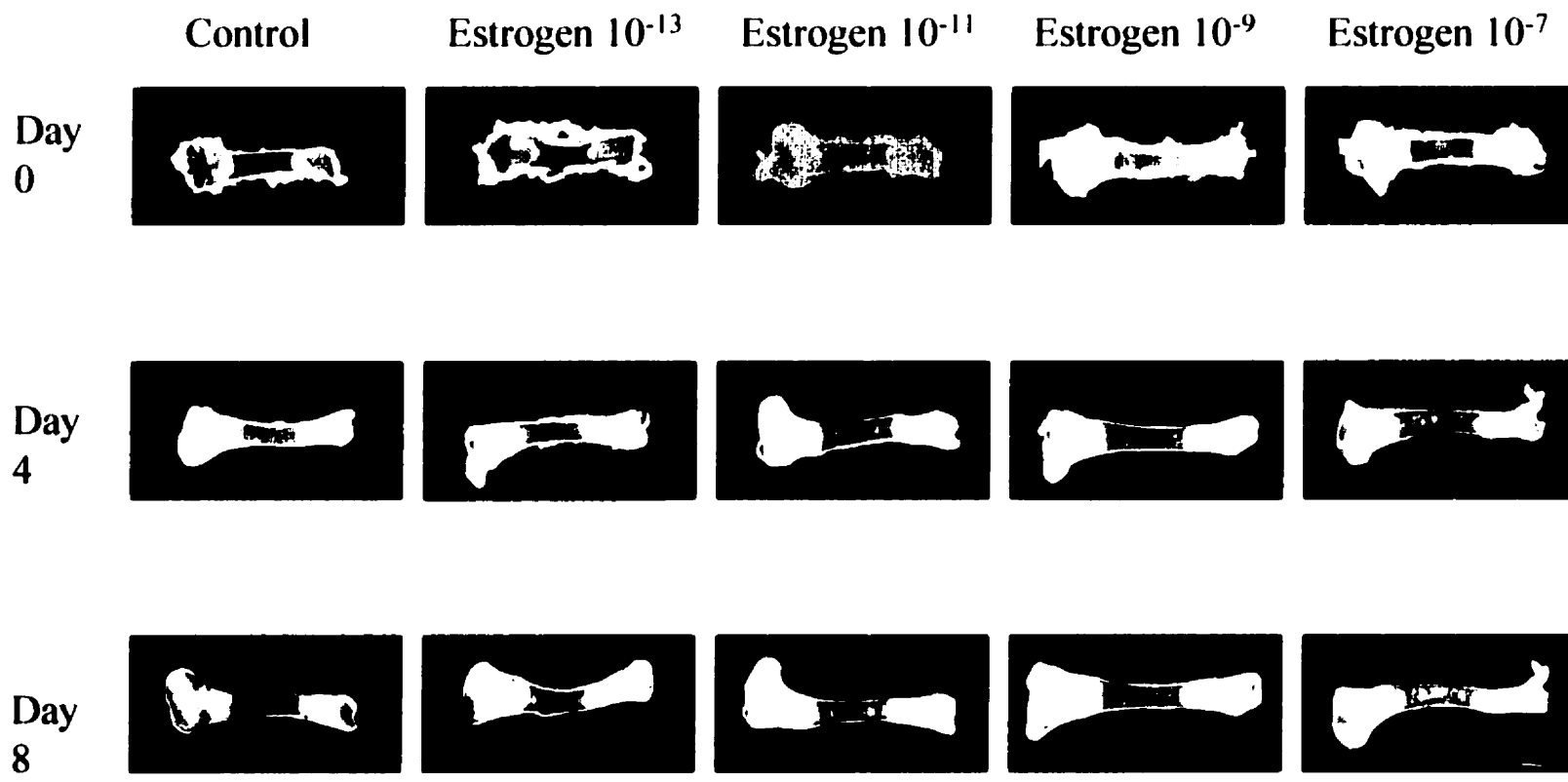
Figure 3-4

Scale = 2mm

Figure3-5: Growth of tibiae in organ culture with estradiol. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in lengths between the groups in any of the zones. Values are mean ± standard deviation.
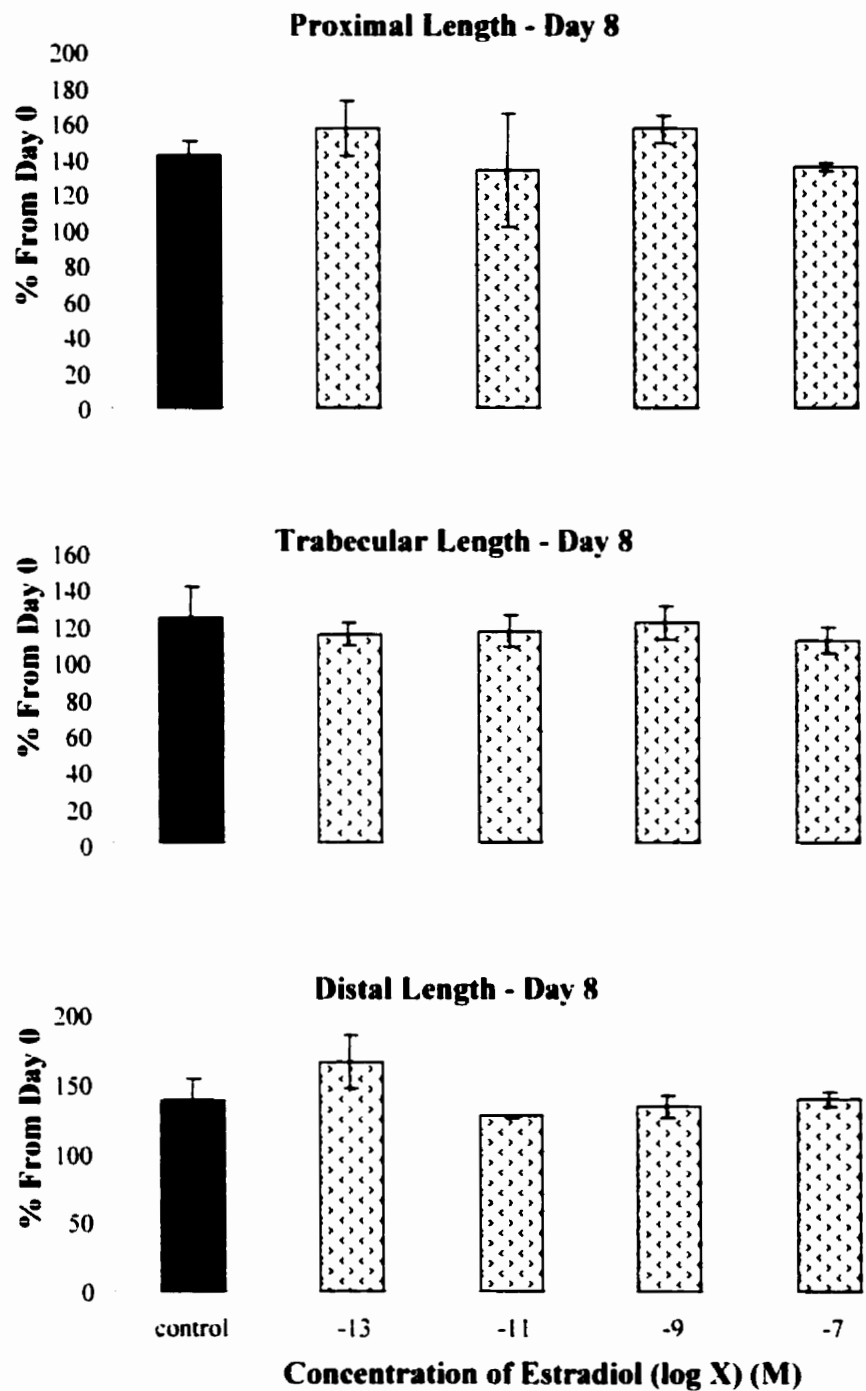
Figure 3-6: Growth of tibiae in organ culture with estradiol. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in lengths between the groups in any of the zones. Values are mean ± standard deviation.

Total Length - Day 4

A

Total Length - Day 8
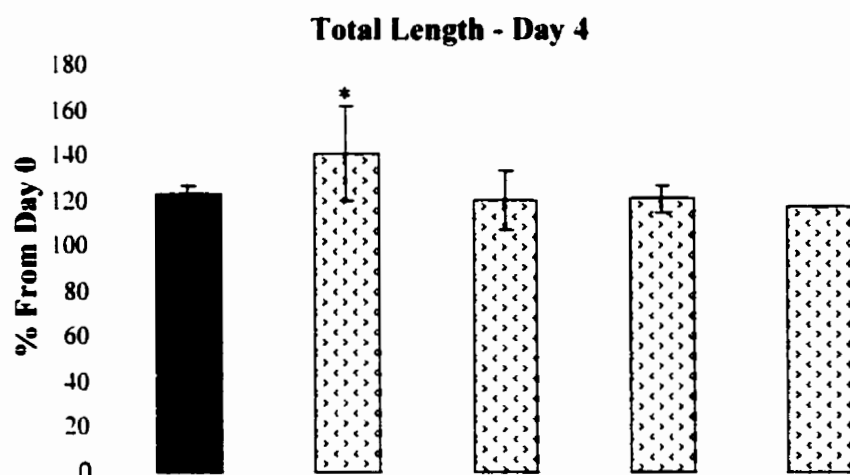
B   **Concentration of Estradiol (log X) (M)**

Figure 3-7: Growth of tibiae in organ culture with estradiol. Lengths of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is a significant increase in total length at day 4 for culture with 10^{-13} M compared to control. There is no significant difference in total length between the other groups. Values are mean ± standard deviation. * indicates statistical significance from control. $p < 0.05$.

Figure 3-8: Growth of tibiae in organ culture with estradiol. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in areas between the groups. Values are mean ± standard deviation.

Figure 3-9: Growth of tibiae in organ culture with estradiol. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 8. There is a significant increase in trabecular area at day 4 for culture with 10^{-13} M compared to control. There is no significant difference in areas between the other groups. Values are mean ± standard deviation. * indicates statistical significance from control. $p<0.05$.

Figure 3-10: Growth of tibiae in organ culture with estradiol. Areas of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is a significant increase in total area at day 4 for culture with 10^{-13} M compared to control. There is no significant difference in total area between the other groups. Values are mean ± standard deviation. * indicates statistical significance from control. $p < 0.05$.

| Control | Estrogen 10^{-13} | Estrogen 10^{-11} | Estrogen 10^{-9} | Estrogen 10^{-7} |

Day 0

Day 4

Day 8

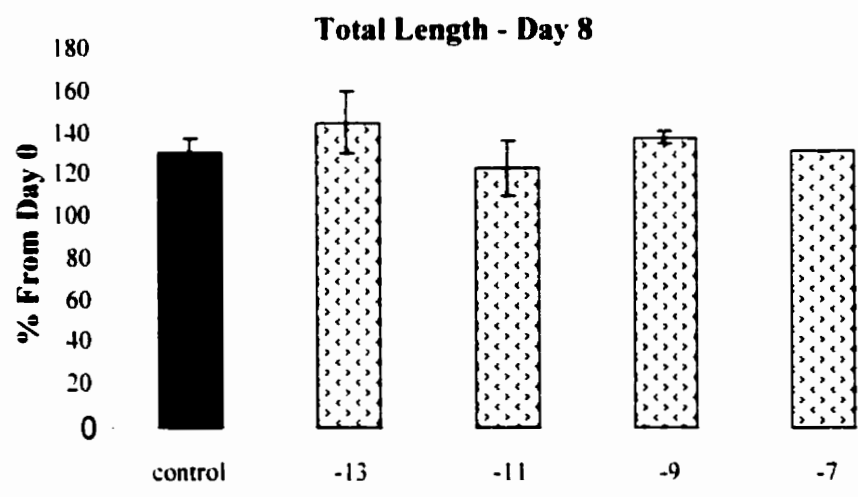**Figure 3-11**

Scale = 2mm

61

Figure 3-12: Growth of femurs in organ culture with estradiol. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in length between the groups. Values are mean ± standard deviation.
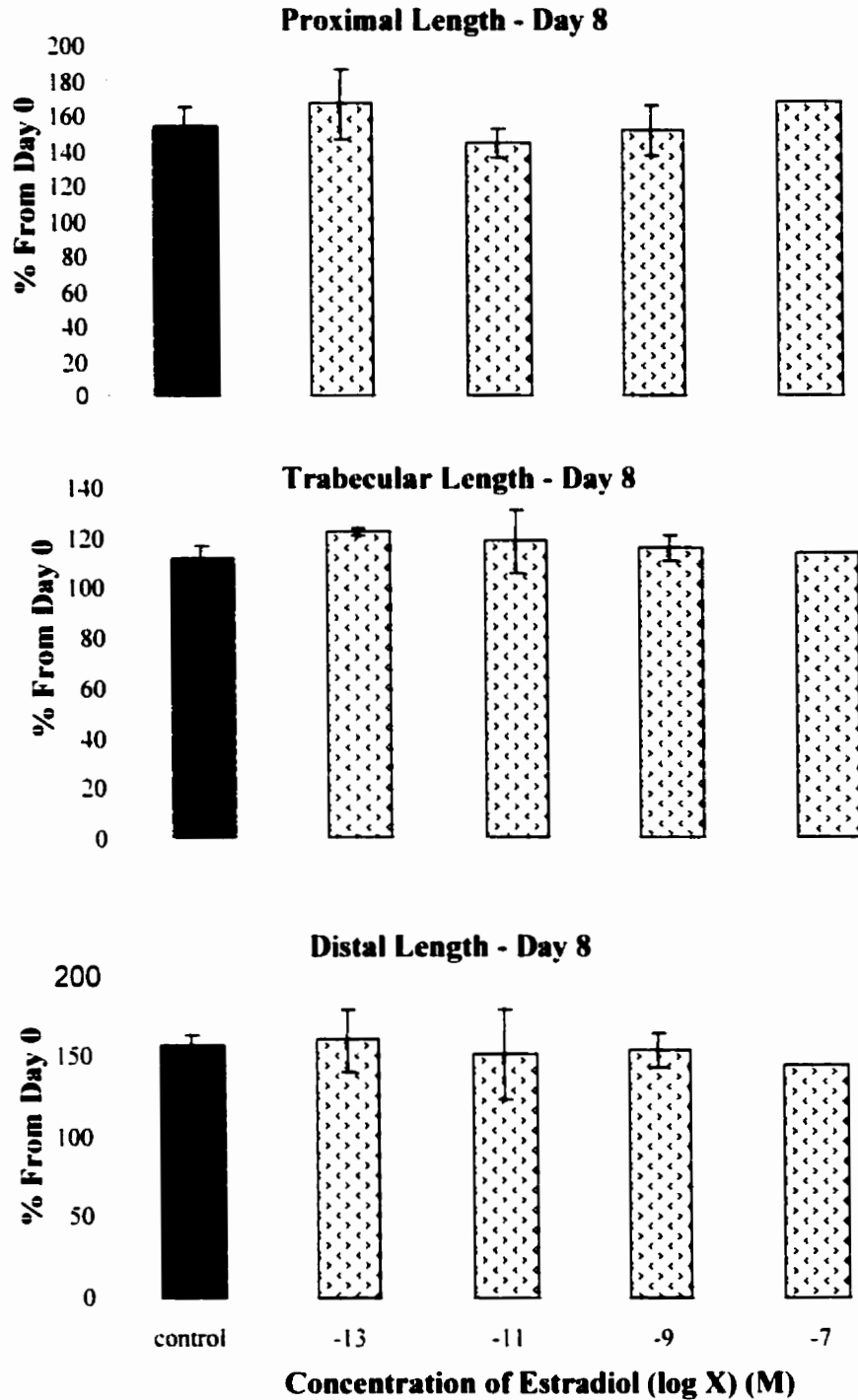
Figure 3-13: Growth of femurs in organ culture with estradiol. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 8. There is no significant difference in length between the groups. Values are mean ± standard deviation.

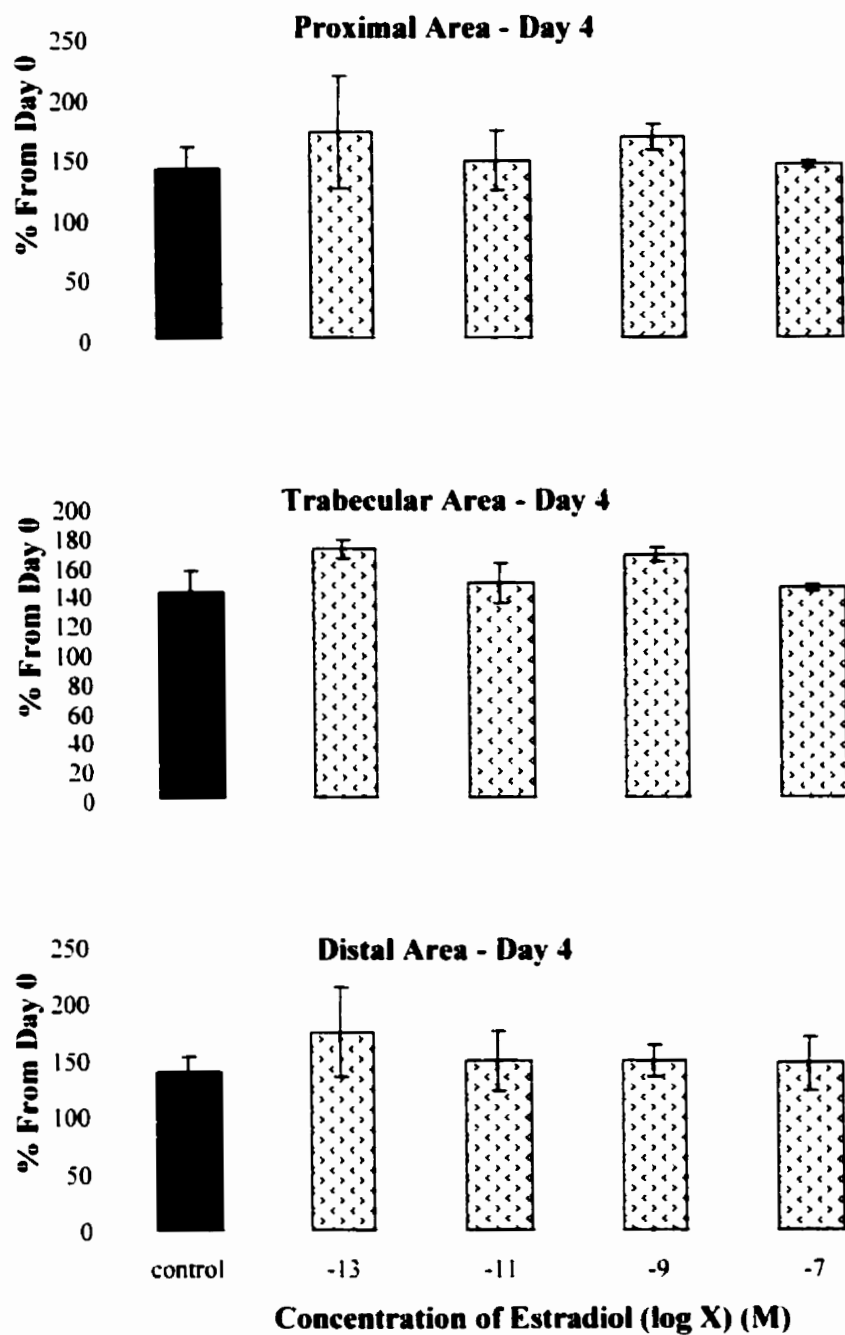**Figure 3-14:** Growth of femurs in organ culture with estradiol. Lengths of total bone were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in total length between the groups. Values are mean ± standard deviation.

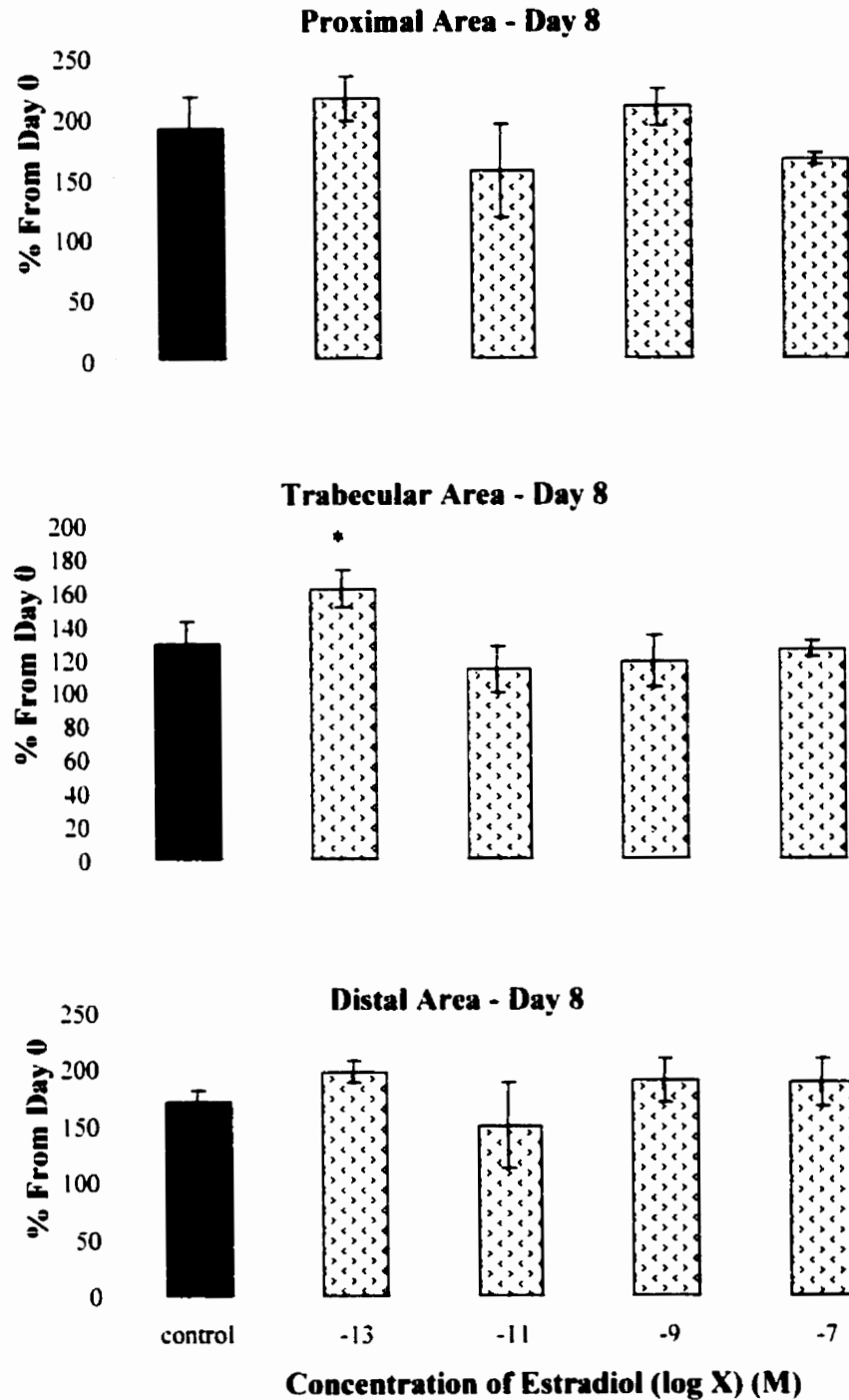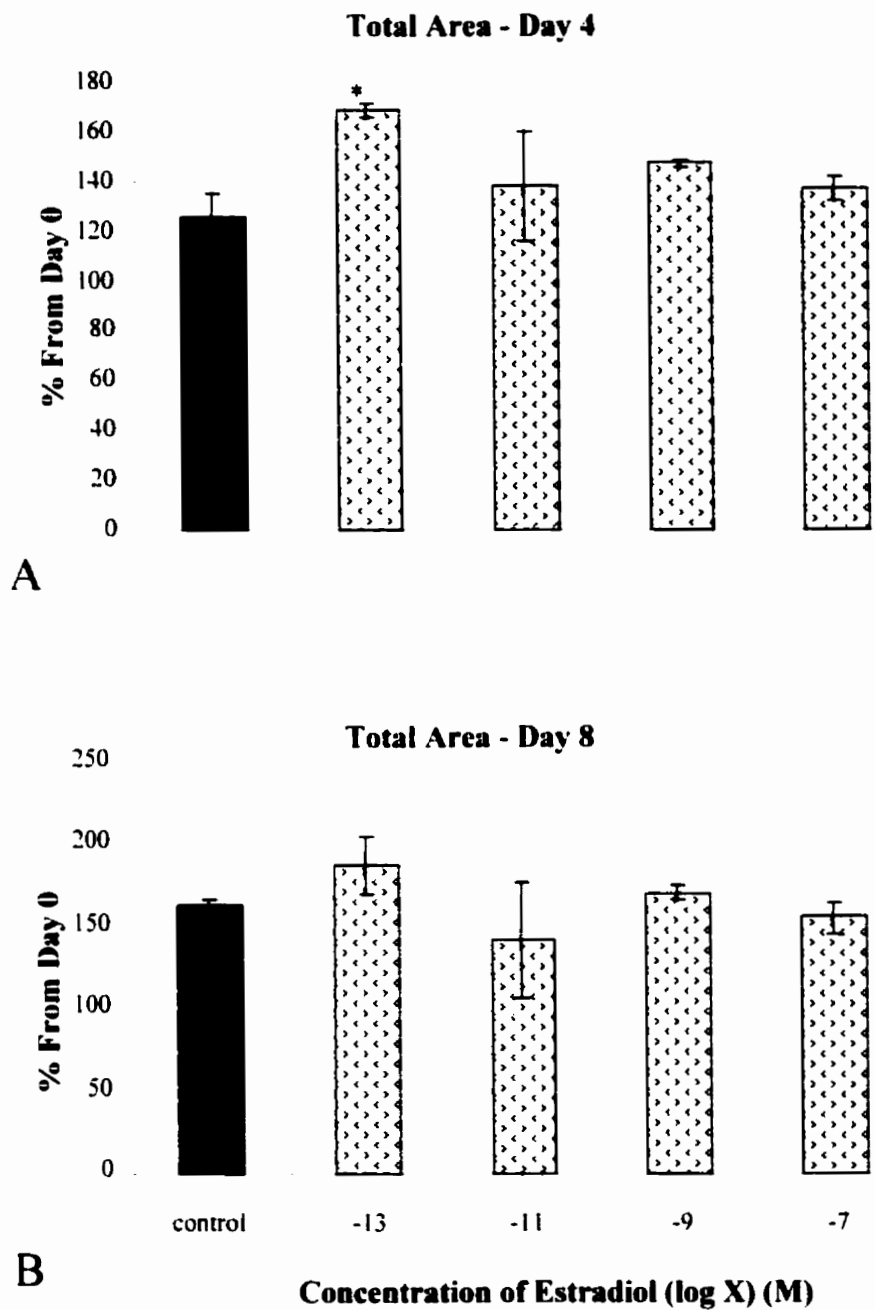**Figure 3-15:** Growth of femurs in organ culture with estradiol. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in areas between the groups. Values are mean ± standard deviation.
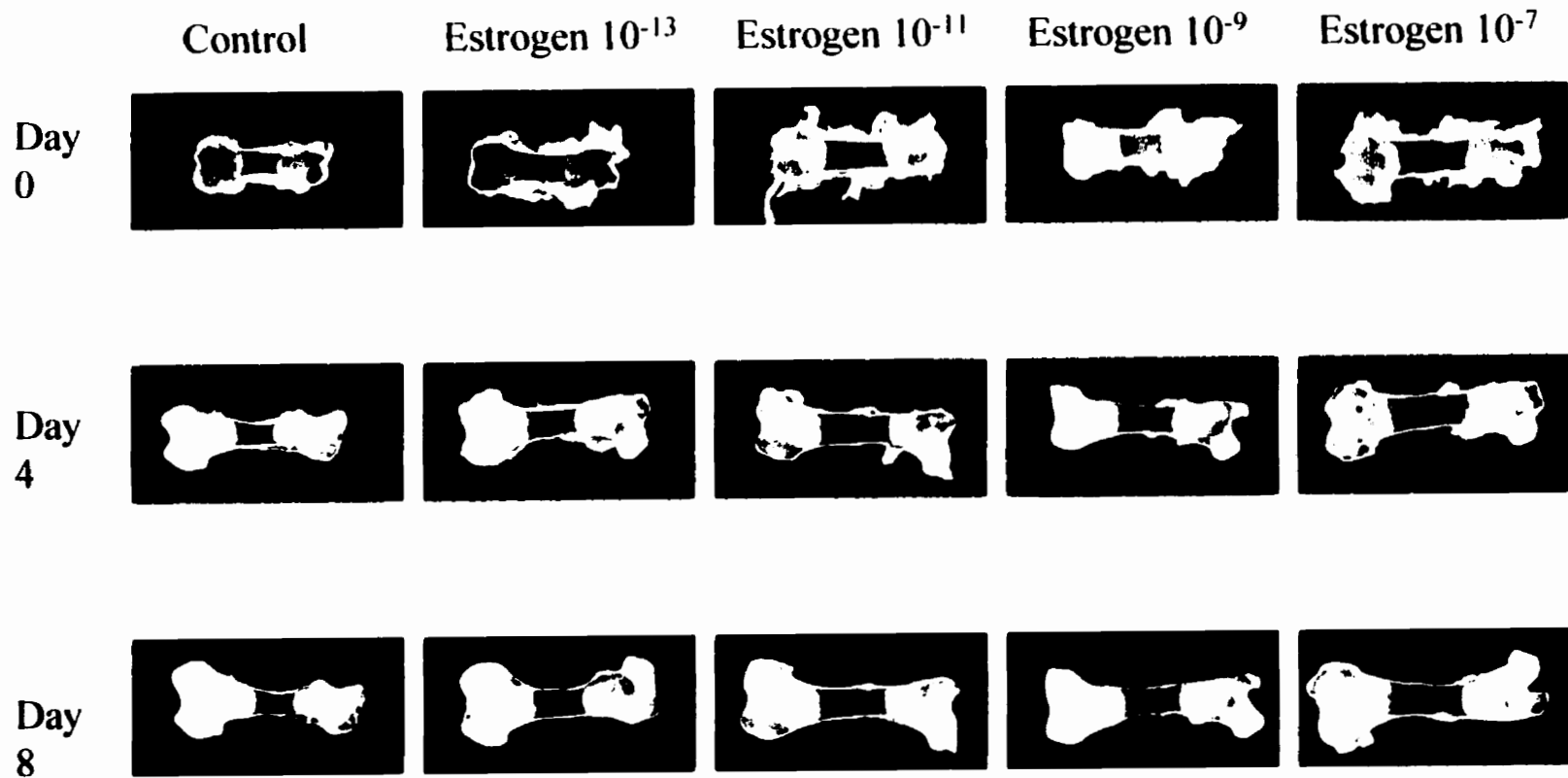
Figure 3-16: Growth of femurs in organ culture with estradiol. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in areas between the groups. Values are mean ± standard deviation.

Figure 3-17: Growth of femurs in organ culture with estradiol. Areas of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is no significant difference in total area between the groups. Values are mean ± standard deviation. * indicates statistical significance from control. $p<0.05$.

Figure 3-18

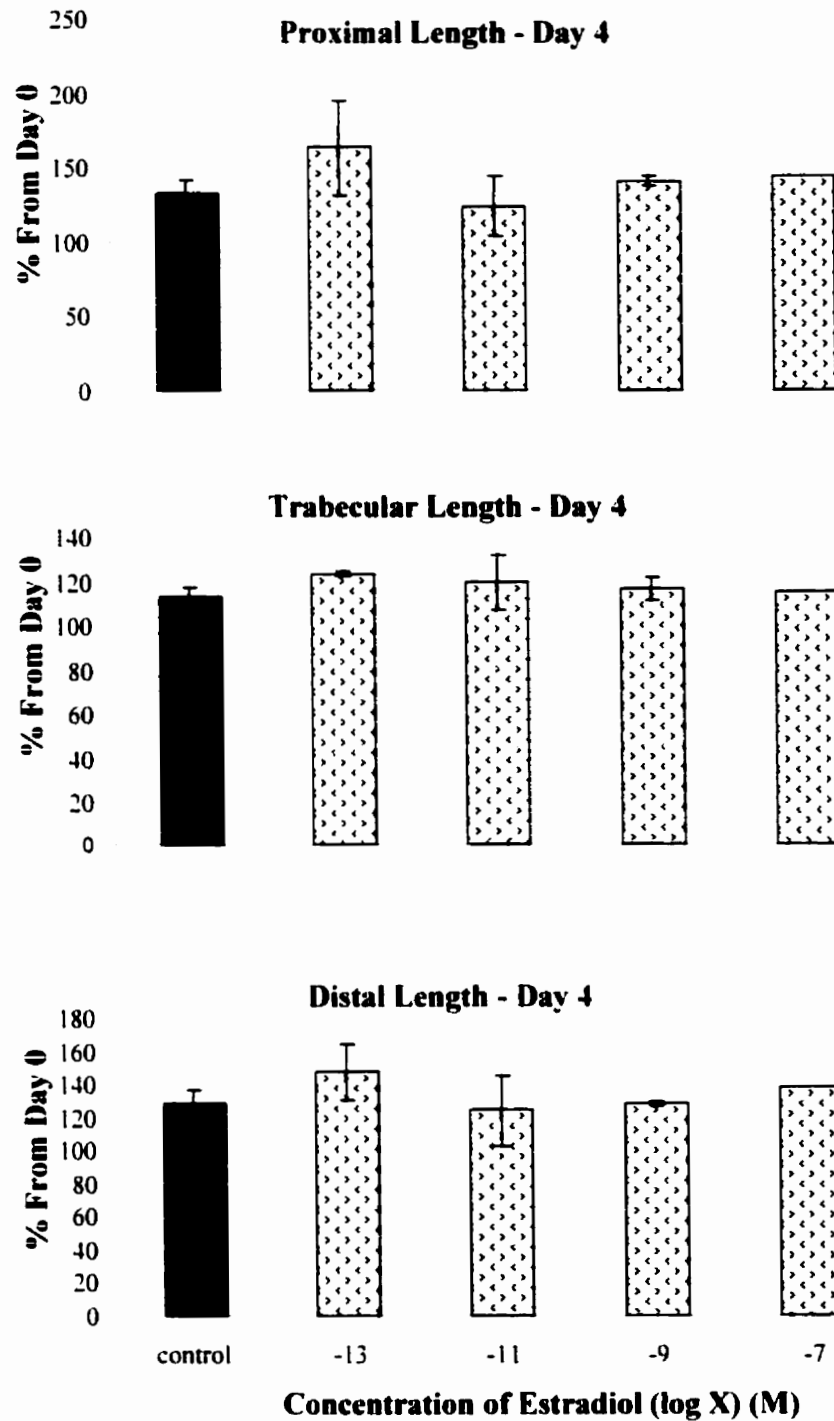68
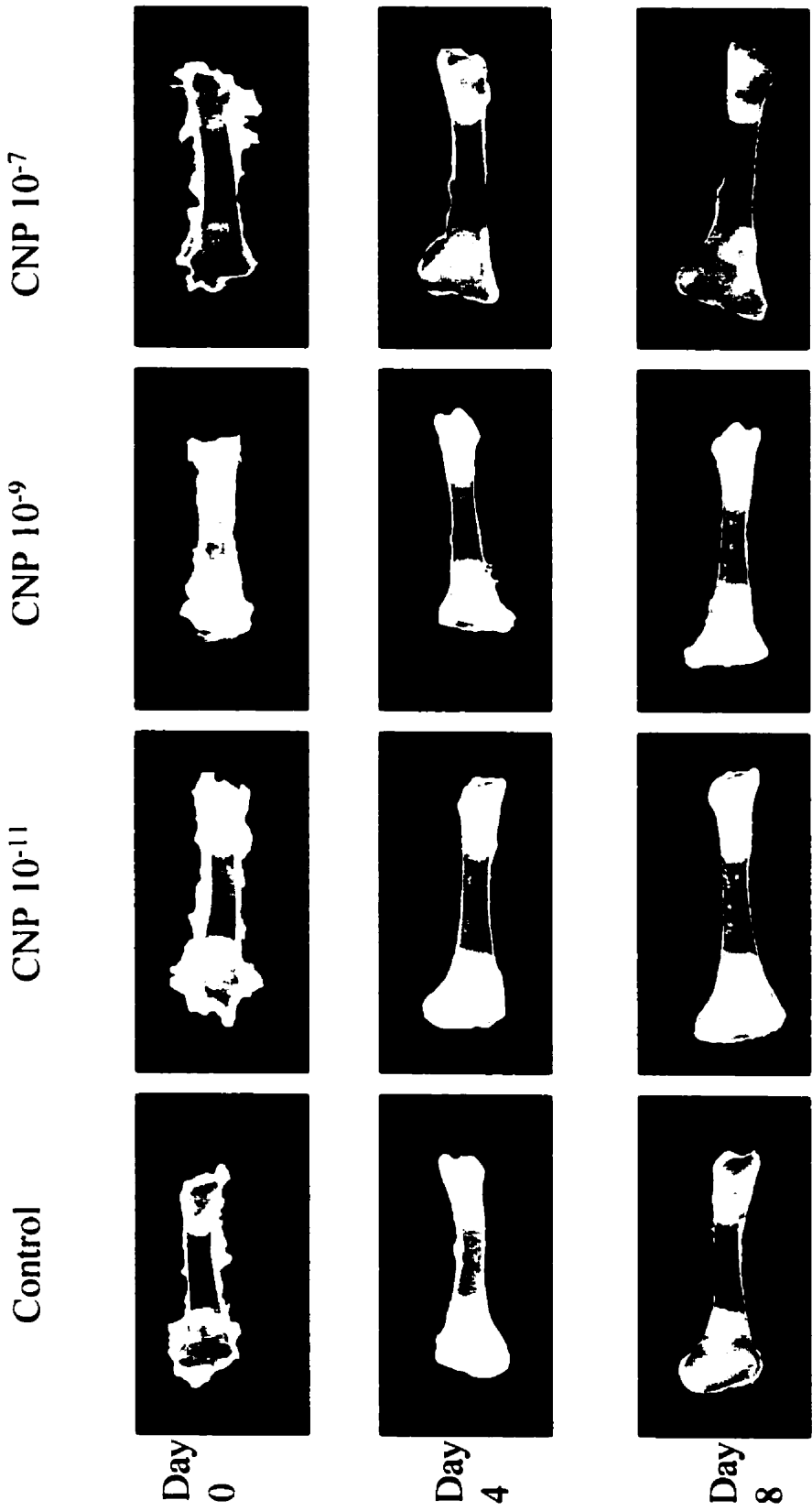
trabecular. distal. or total length at day 4 or day 8 (Figure 3-19, 3-20. and 3-21) at any of the doses of CNP compared to control. At day 4 and day 8. a significant increase in proximal. distal and total areas was seen between control and CNP at 10^{-7} M (Figure 3-22. 3-23. and 3-24). An increase in trabecular area was not evident.

In femurs. a similar trend was demonstrated. Photomicrographs taken at day 0. day 4 and day 8 (Figure 3-25) were used to measure lengths and areas. No statistical significance was seen in proximal. trabecular. distal. or total length at day 4 or day 8 (Figure 3-26, 3-27. and 3-28) at any of the doses of CNP compared to control. However. unlike in the tibiae. no significant increases were seen at day 4 in any of the doses or any areas. At day 8. a significant increase of in proximal. distal and total areas was seen between control and CNP at 10^{-7} M (Figure 3-29, 3-30. and 3-31). An increase in trabecular area was not evident.

3.3.4 Morphological Studies

Measurement of Proliferative and Hypertrophic Zones

Toluidene blue stains acidic cell components. such as the nucleus and bony spicules leaving the rest of the stain a light magenta colour. Comparison of the 10^{-11}, 10^{-9}. and 10^{-7} M sections at 4X magnification does not reveal any morphological differences (Figures 3-32 – 3-35).

CNP had negligible effects on the length of the proliferative and hypertrophic zones (as a per cent of total epiphyseal length) in both the proximal and distal epiphyseal areas of the femoral sections (Figure 3-36 and 3-37).
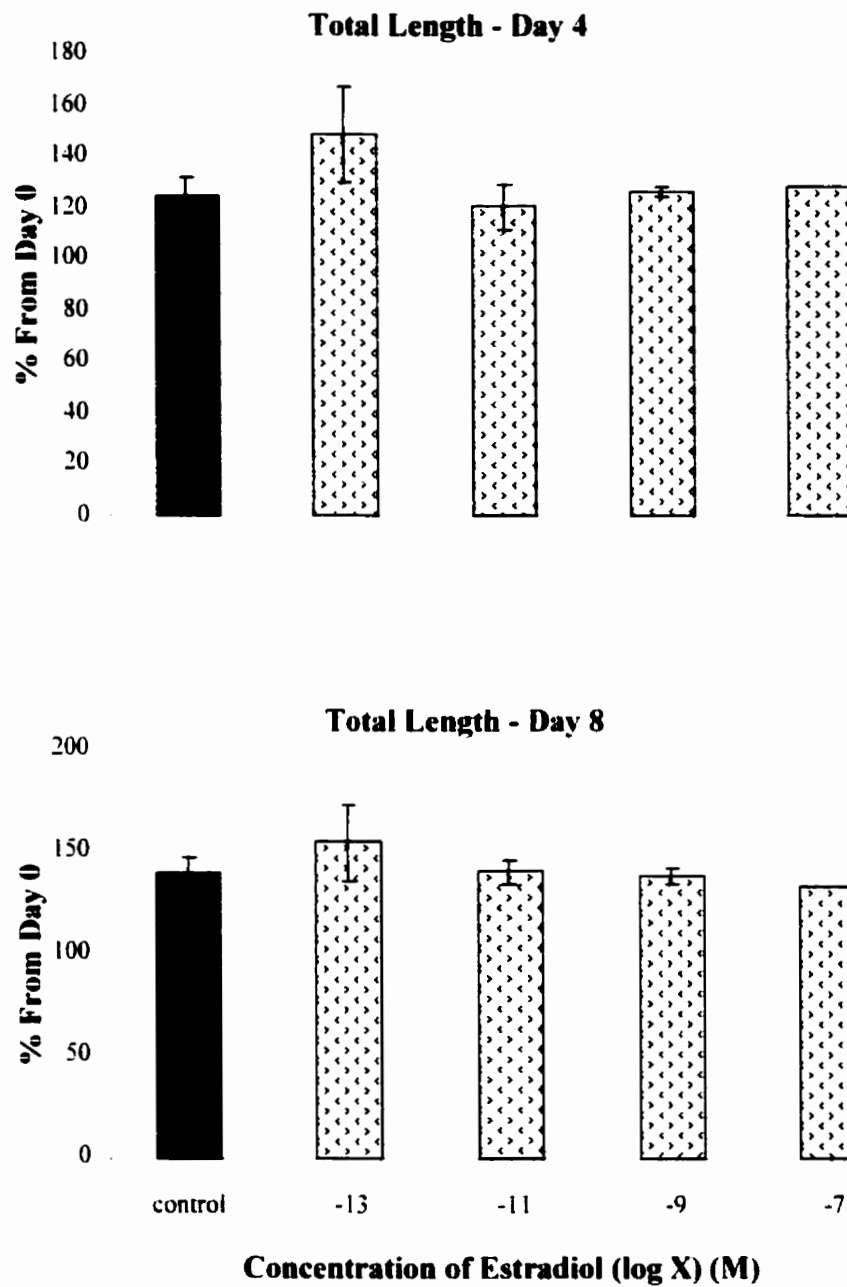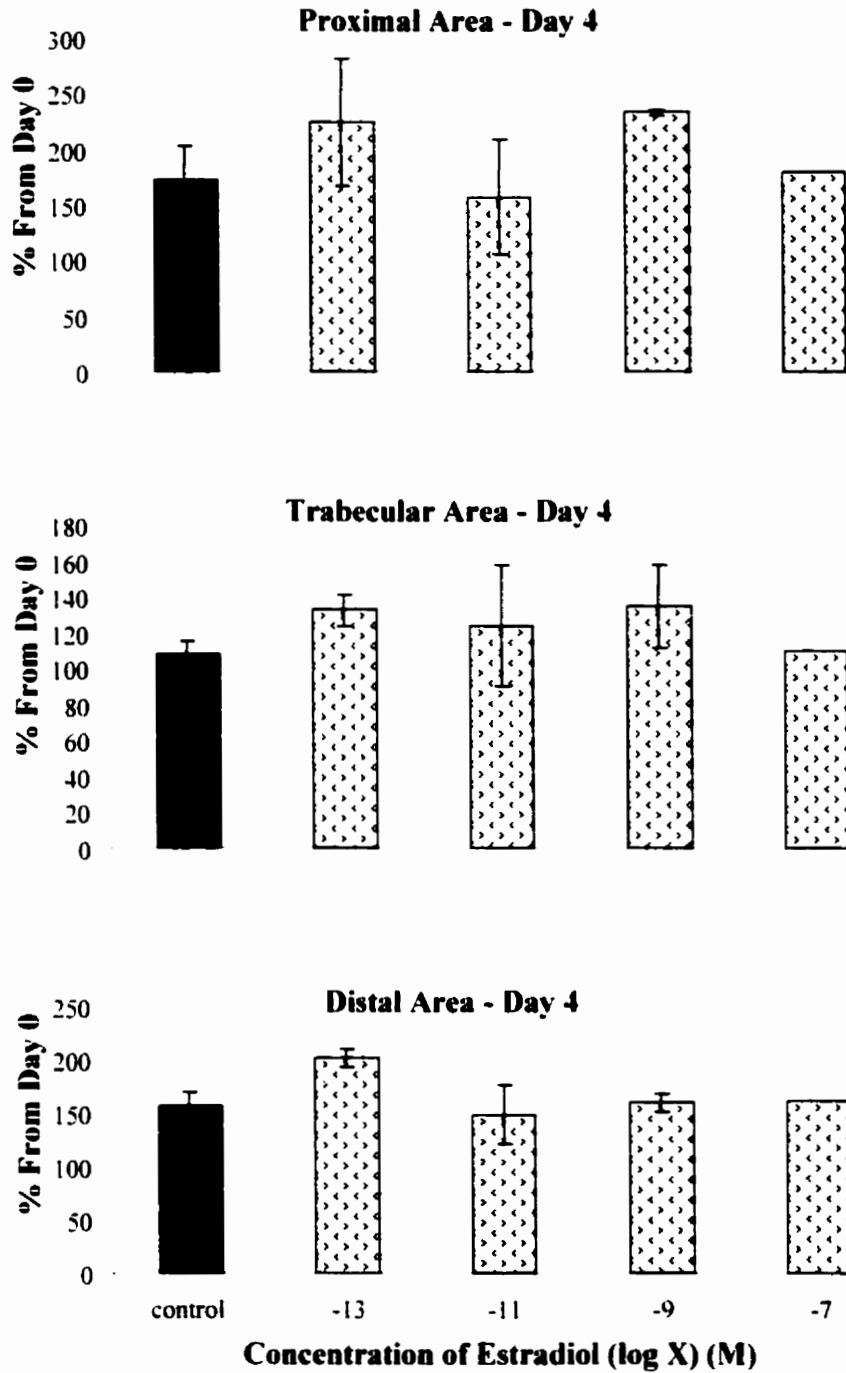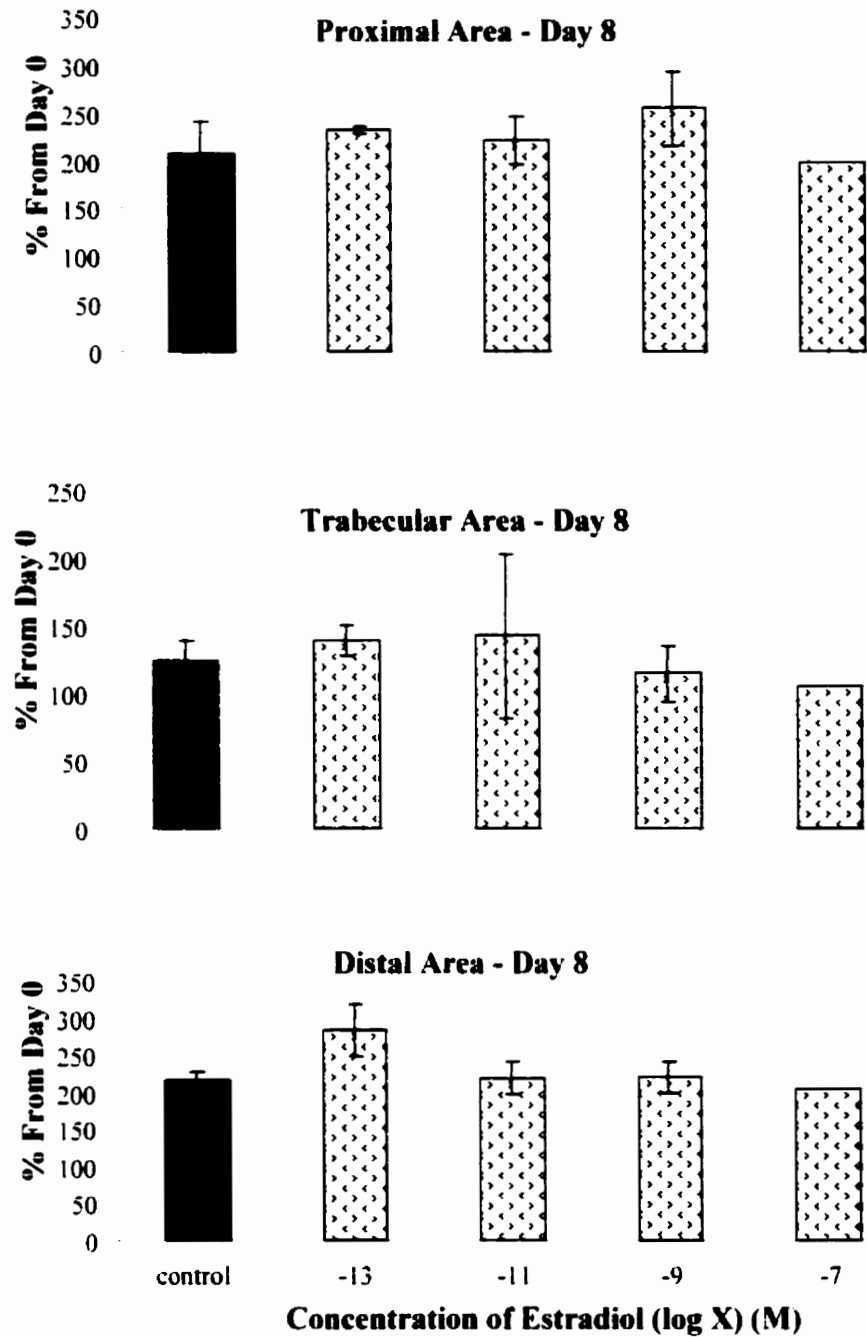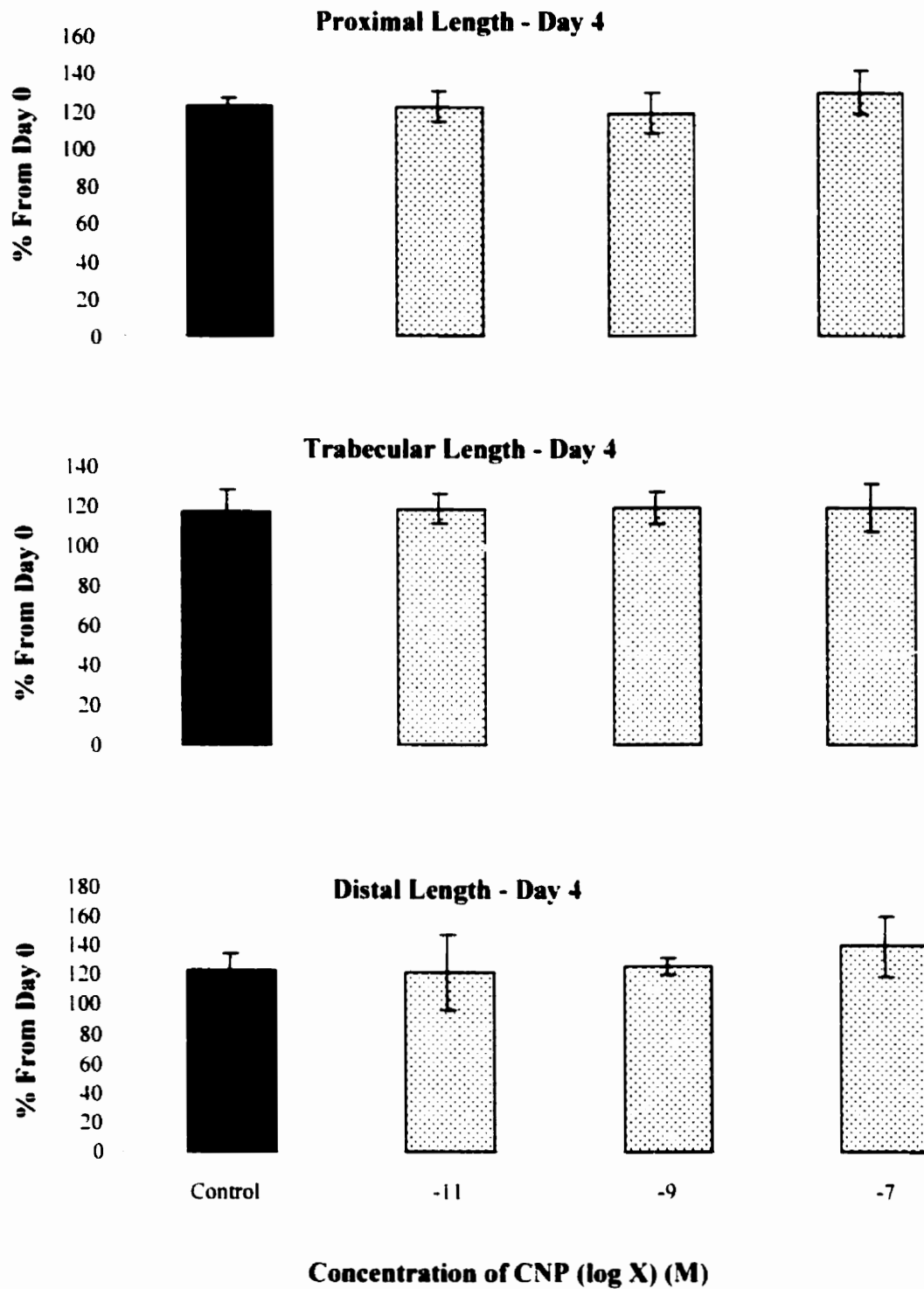
Figure 3-19: Growth of tibiae in organ culture with CNP. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in length between the groups in any of the zones. Values are mean ± standard deviation.
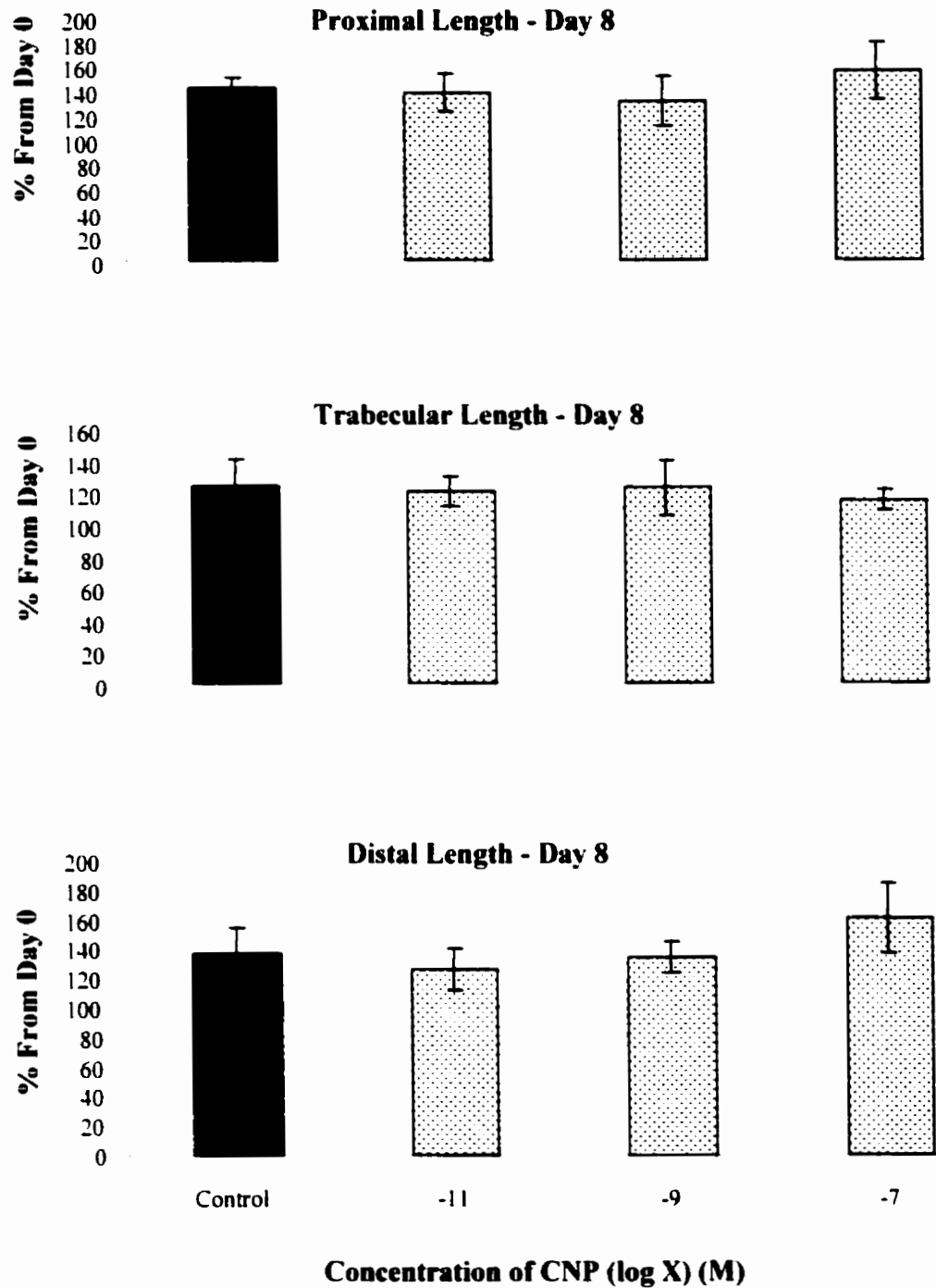
Figure 3-20: Growth of tibiae in organ culture with CNP. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 8. There is no significant difference in length between the groups in any of the zones. Values are mean ± standard deviation.
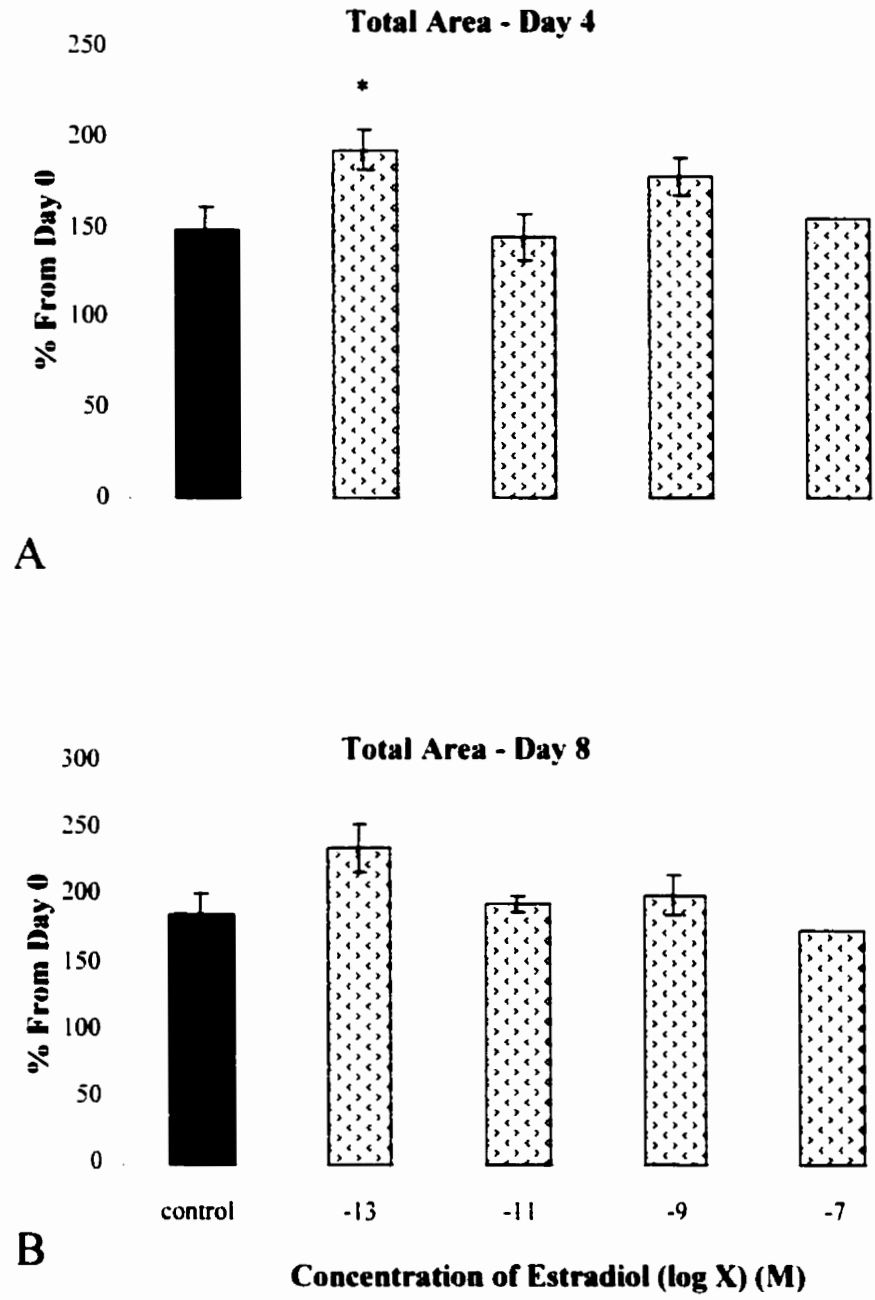
Figure 3-21: Growth of tibiae in organ culture with CNP. Lengths of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is no significant difference in total length between the groups. Values are mean ± standard deviation.

Figure 3-22: Growth of tibiae in organ culture with CNP. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is a significant increase in area in both the proximal and distal areas at 10^{-7} M compared to control. Values are mean ± standard deviation. * indicates statistical significance from control. $p < 0.05$.

Figure 3-23: Growth of tibiae in organ culture with CNP. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is a significant increase in area in both the proximal and distal areas at 10^{-7} M compared to control. Values are mean ± standard deviation. * indicates statistical significance from control. $p < 0.05$.
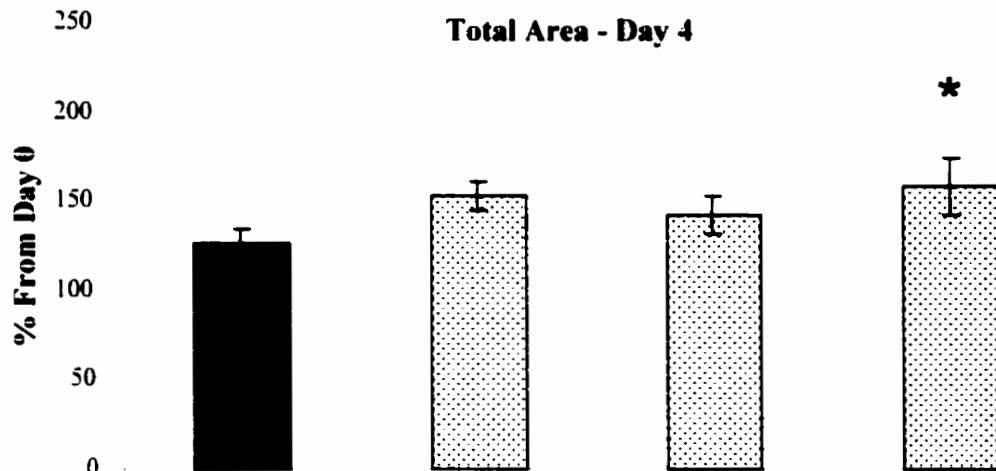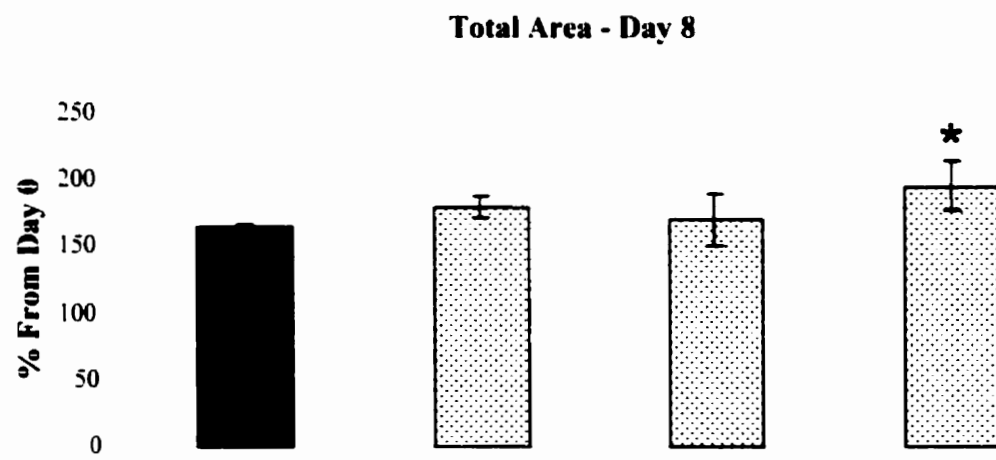
Figure 3-24: Growth of tibiae in organ culture with CNP. Areas of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is a significant increase in area in total area at 10^{-7} M compared to control. Values are mean ± standard deviation. * indicates statistical significance from control. $p<0.05$.

| | Control | CNP 10^{-11} | CNP 10^{-9} | CNP 10^{-7} |

Day 0

Day 4

Day 8
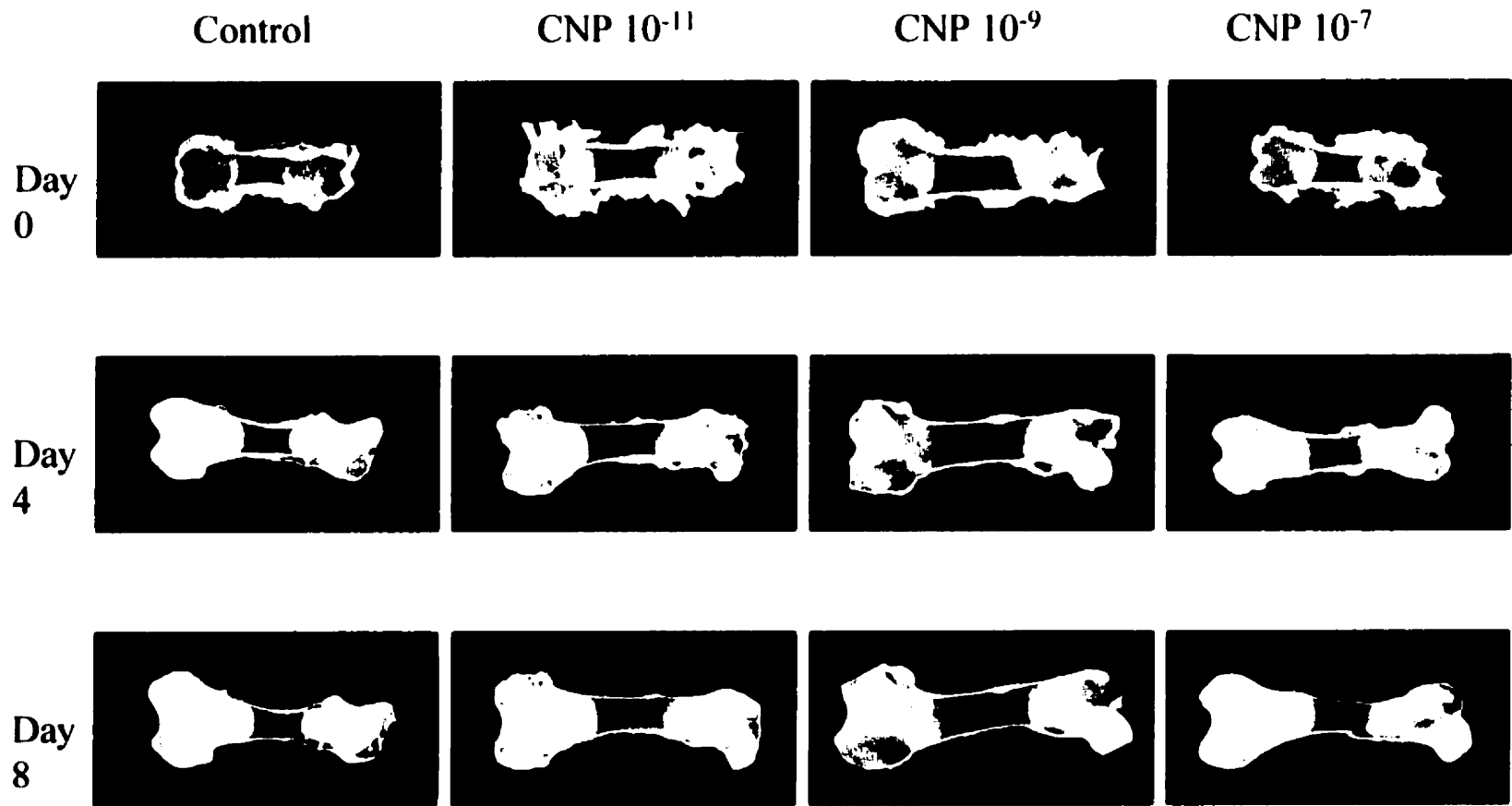
Figure 3-25

Scale = 2mm
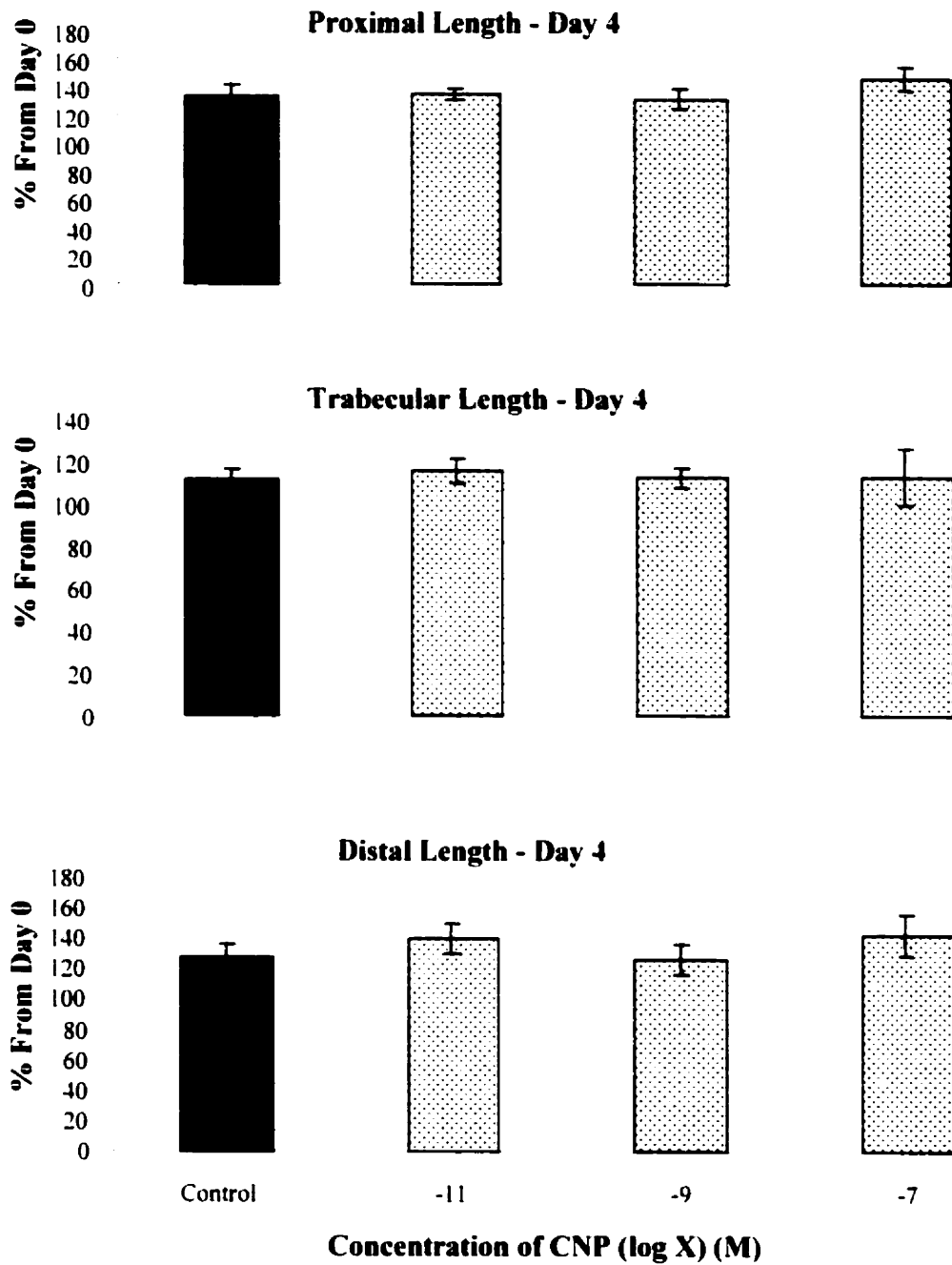
Figure 3-26: Growth of femurs in organ culture with CNP. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in length between the groups in any of the zones. Values are mean ± standard deviation.
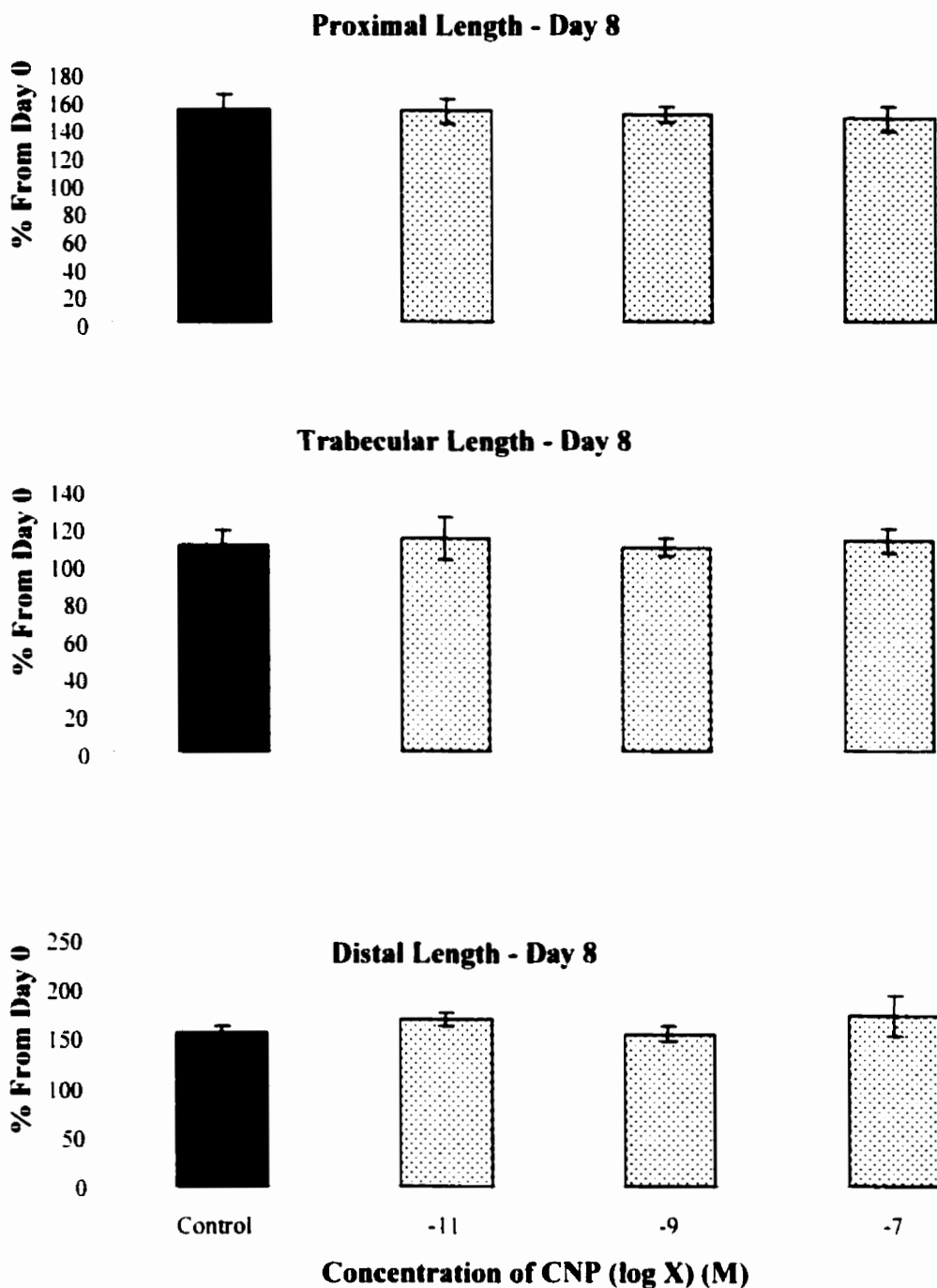
Figure 3-27: Growth of femurs in organ culture with CNP. Lengths of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in length between the groups in any of the zones. Values are mean ± standard deviation.
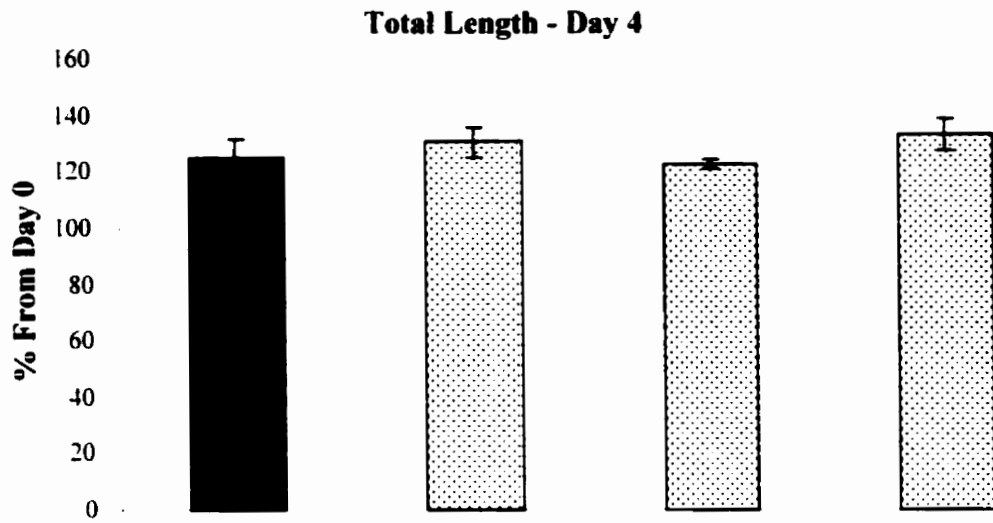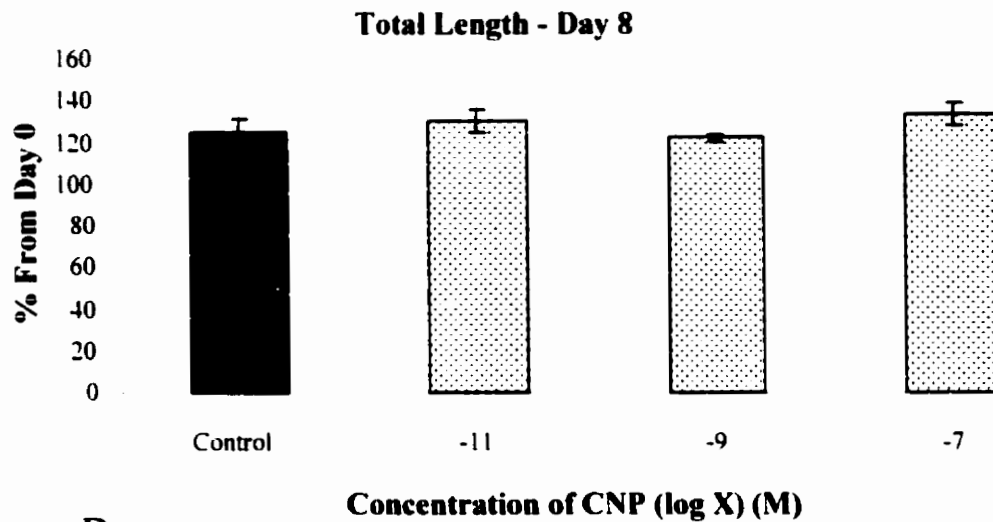
Figure 3-28: Growth of femurs in organ culture with CNP. Lengths of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is no significant difference in total length between the groups. Values are mean ± standard deviation.
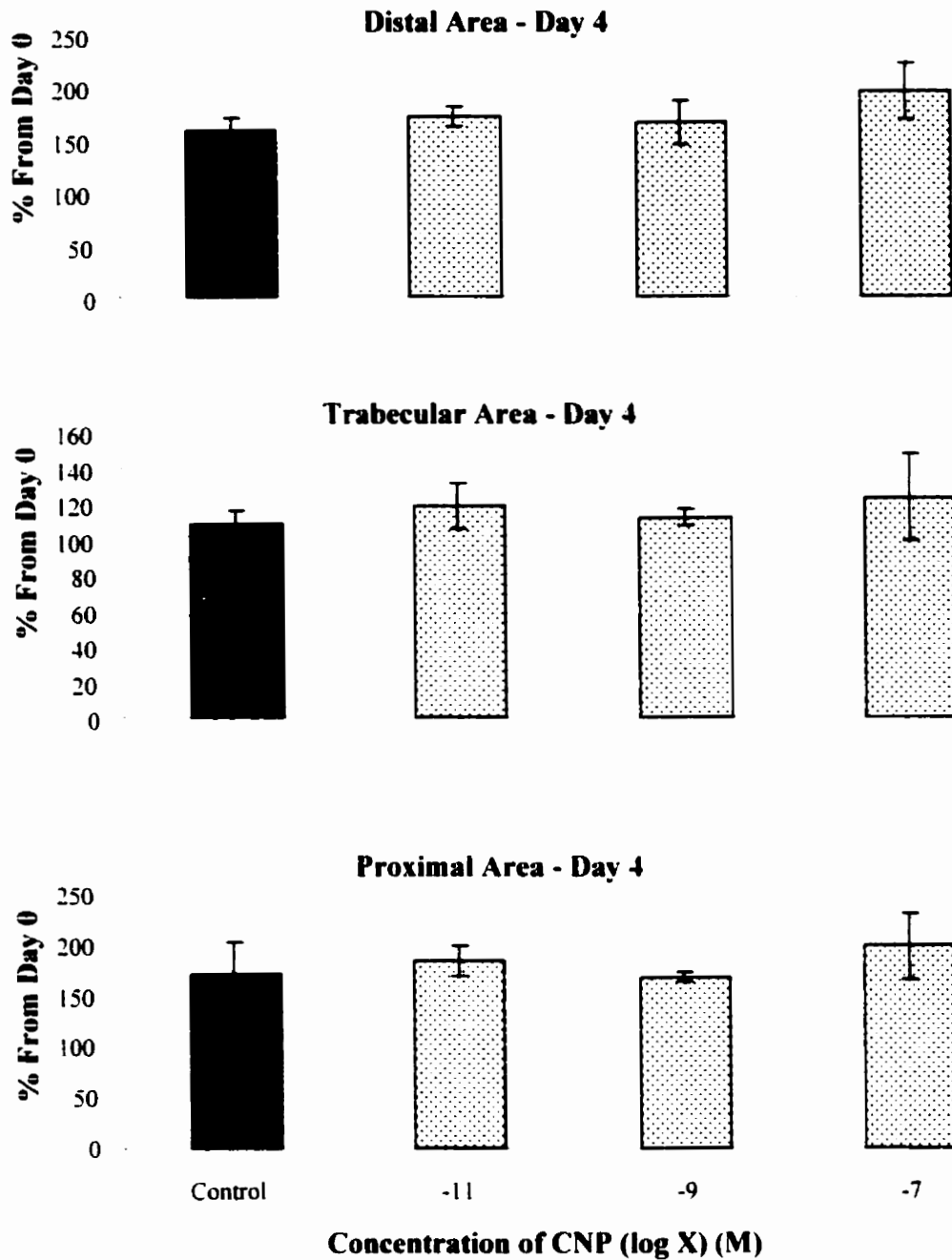
Figure 3-29: Growth of femurs in organ culture with CNP. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 4. There is no significant difference in total length between the groups. Values are mean ± standard deviation.
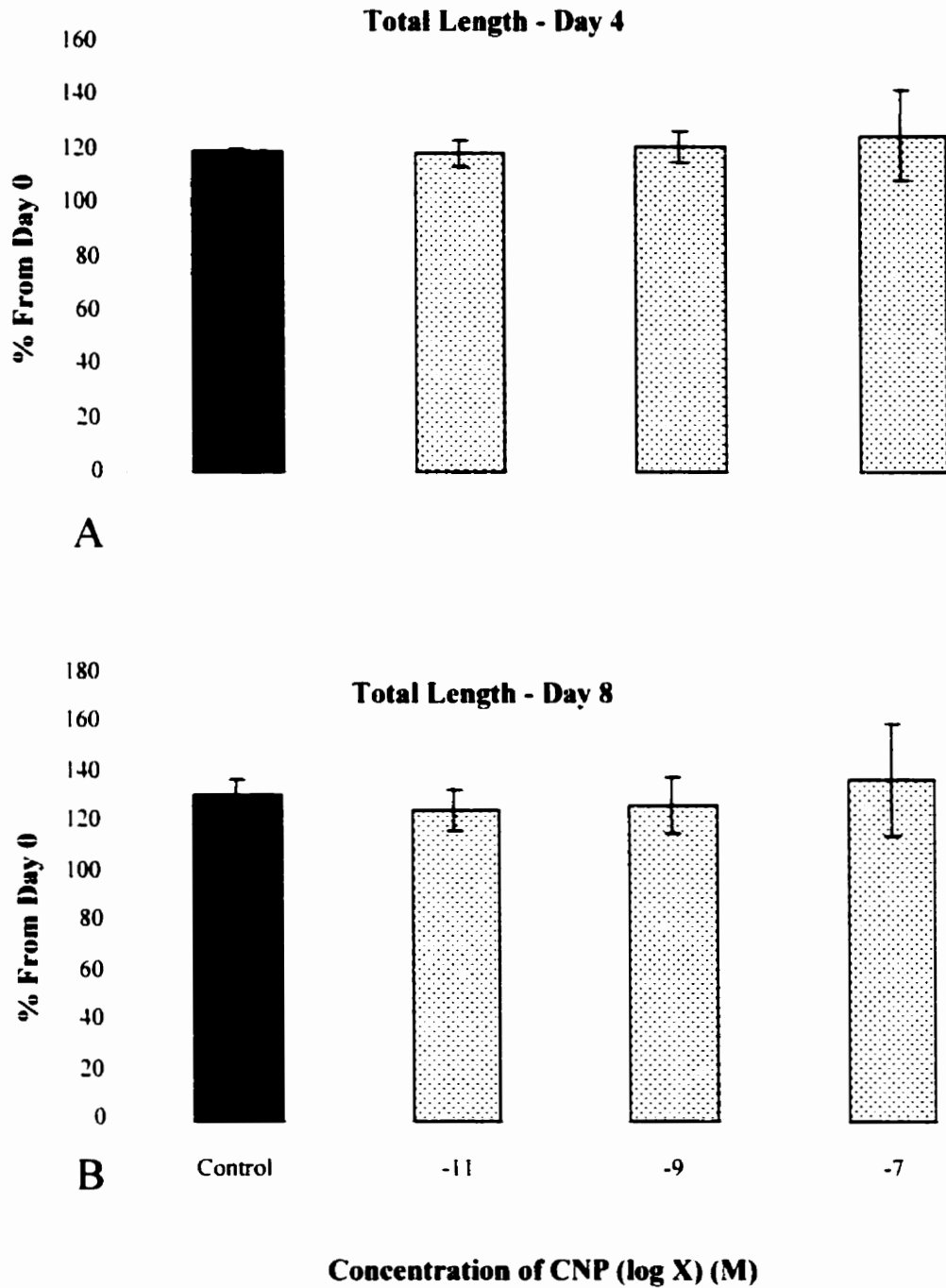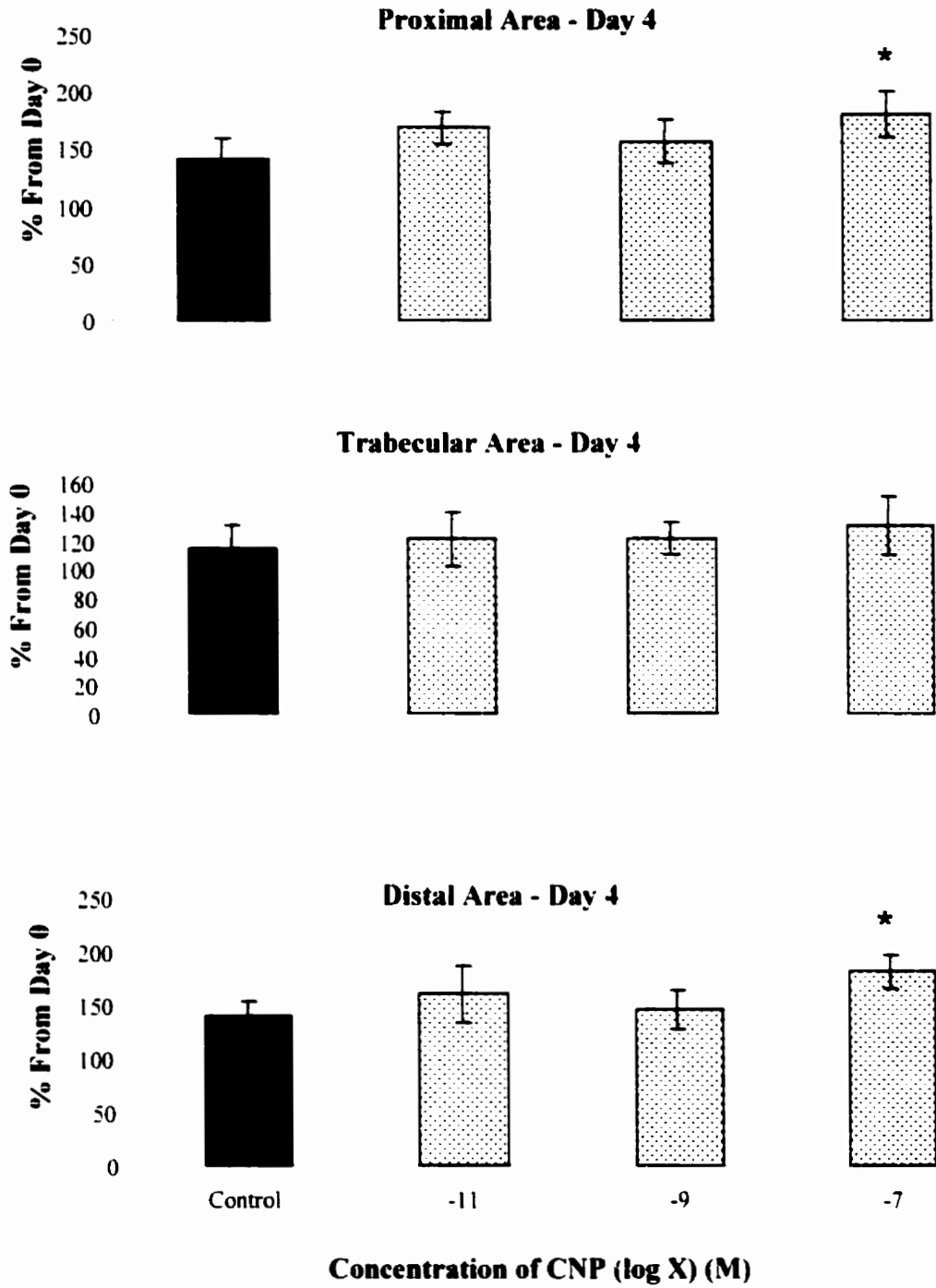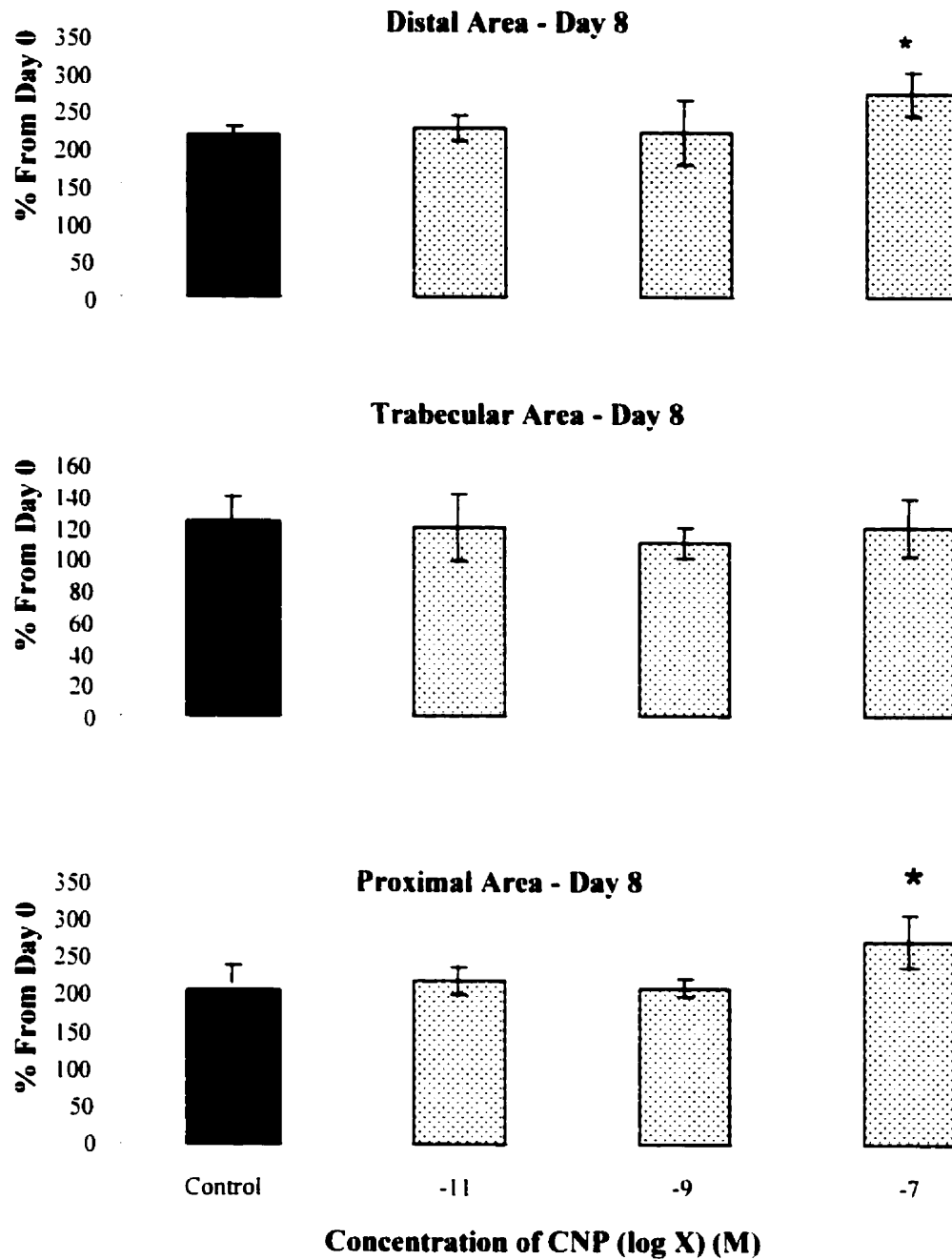
Figure 3-30: Growth of femurs in organ culture with CNP. Areas of indicated zones were measured and calculated as percentage growth from day 0 to day 8. There is a significant increase in area in both the proximal and distal areas at 10^{-7} M compared to control. Values are mean ± standard deviation. * indicates statistical significance from control. p<0.05.
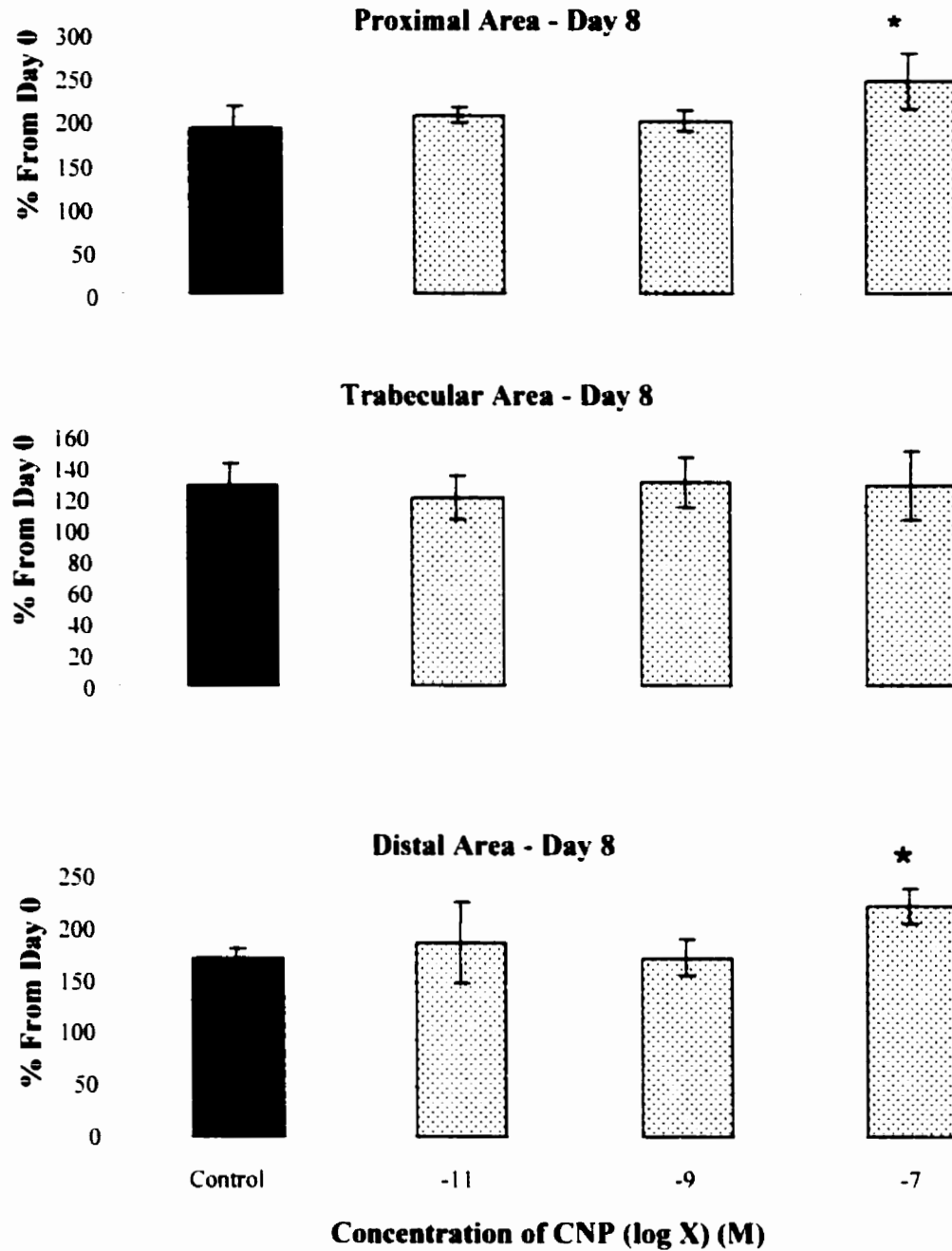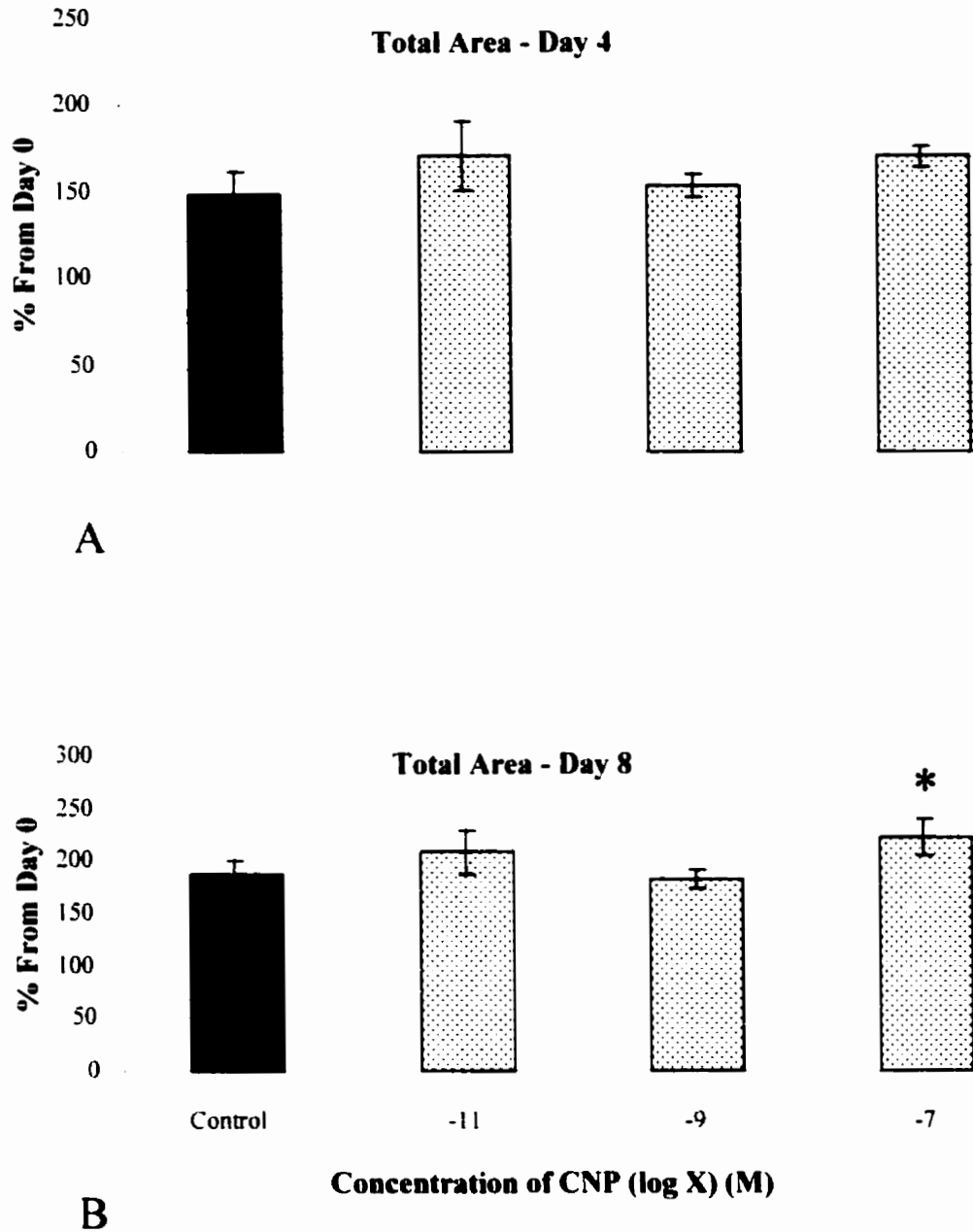
Figure 3-31: Growth of femurs in organ culture with CNP. Areas of total bone were measured and calculated as percentage growth from (A) day 0 to day 4 and (B) day 0 to day 8. There is a significant increase in total area at 10^{-7} M compared to control. Values are mean ± standard deviation. * indicates statistical significance from control, $p < 0.05$.
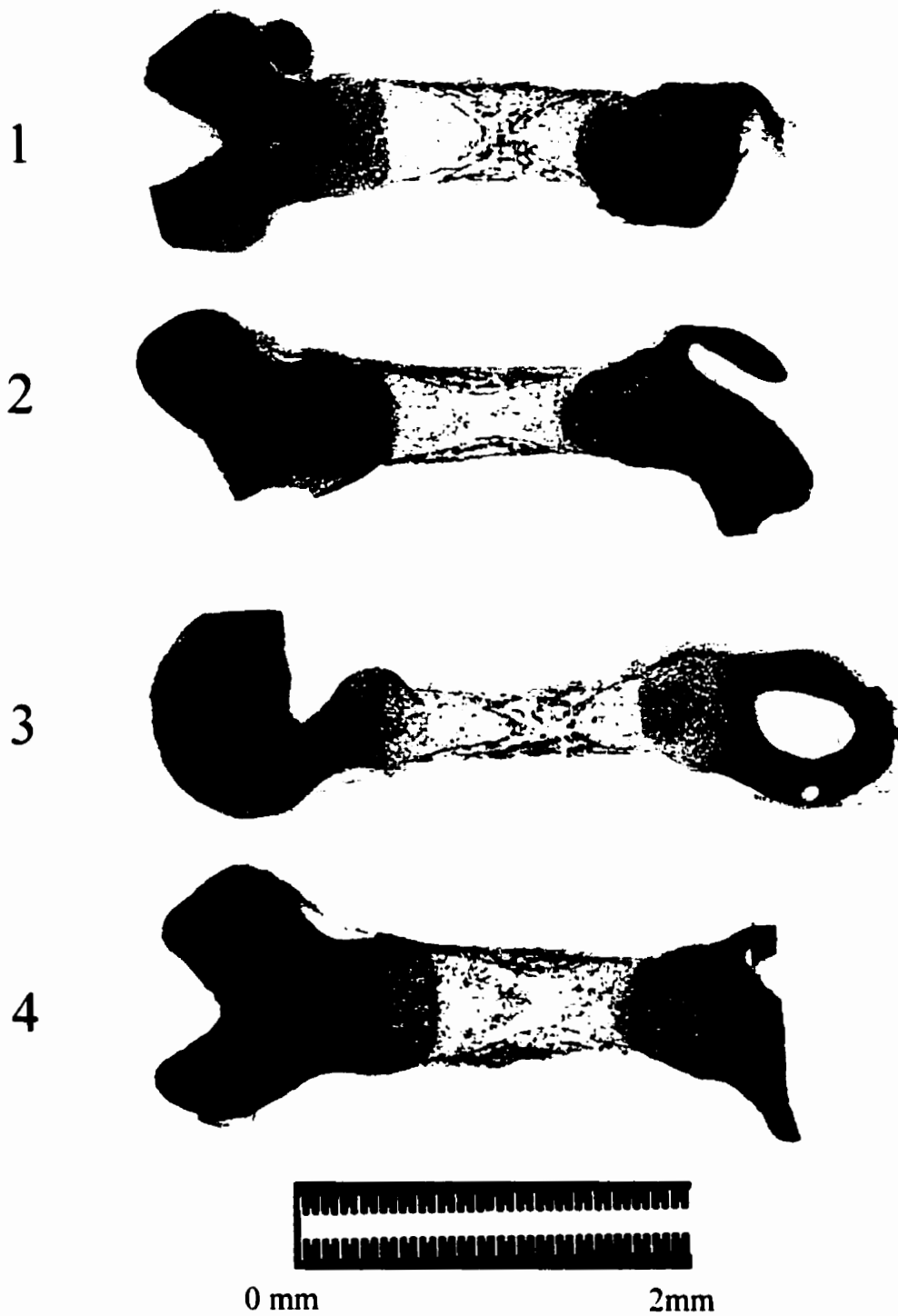
Figure 3-32: Histological sections of four different femurs after 8 days in culture without CNP. Sections are stained in toluidene blue.
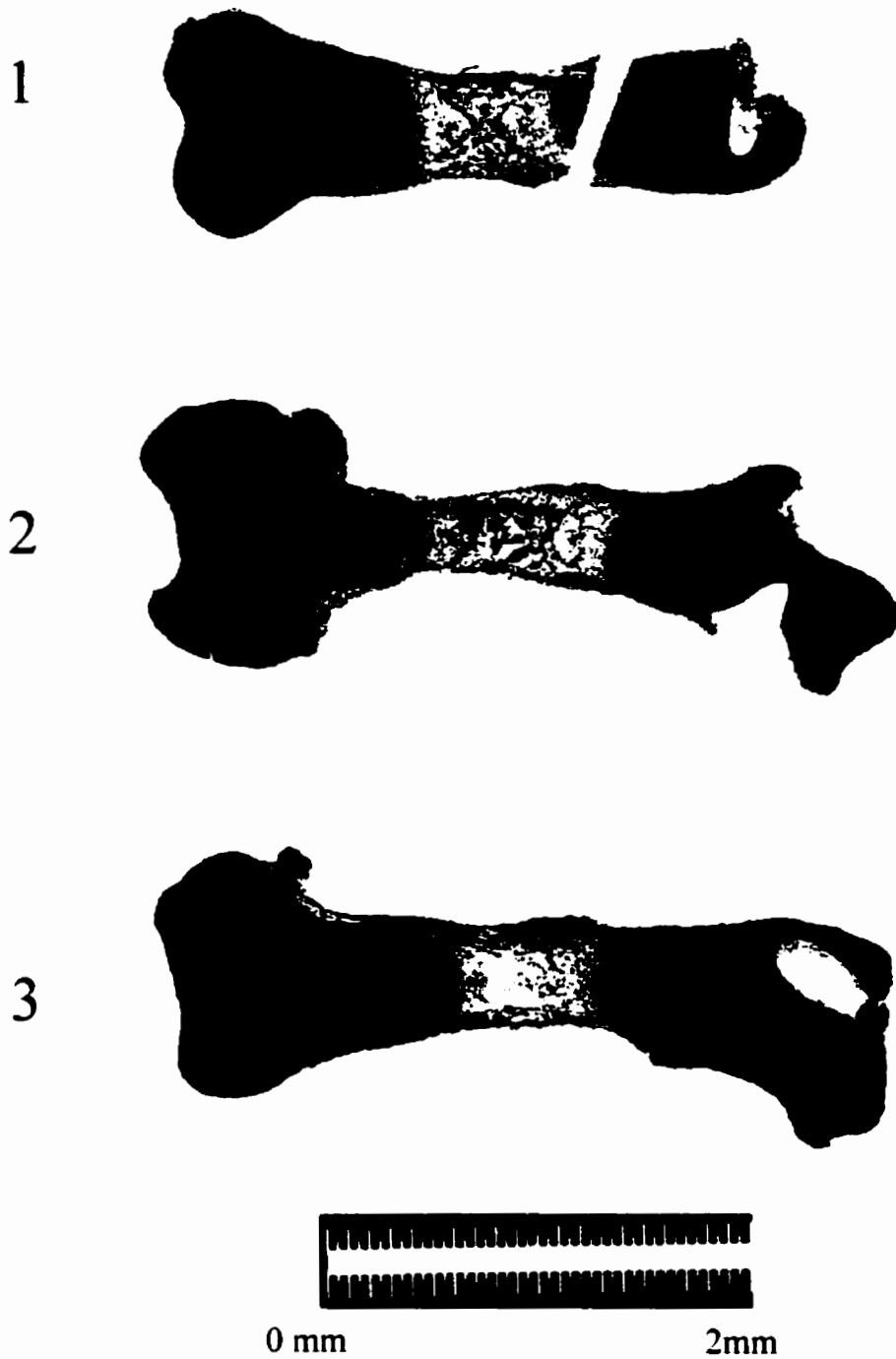
Figure 3-33: Histological sections of three different femurs after 8 days in culture with CNP at 10^{-11} M. Sections are stained in toluidene blue.
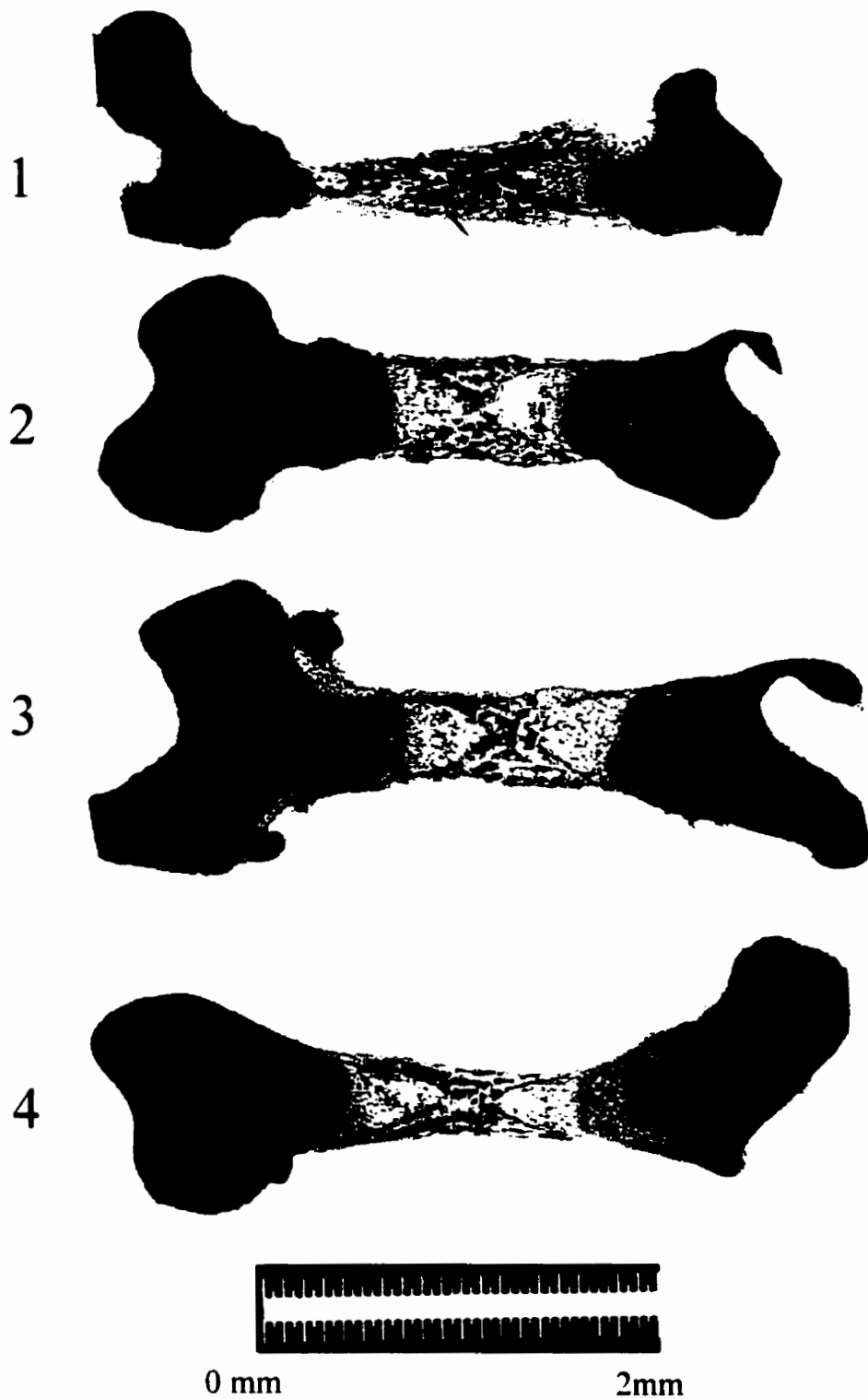
Figure 3-34: Histological sections of four different femurs after 8 days in culture with CNP at 10^{-9} M. Sections are stained in toluidene blue.

85
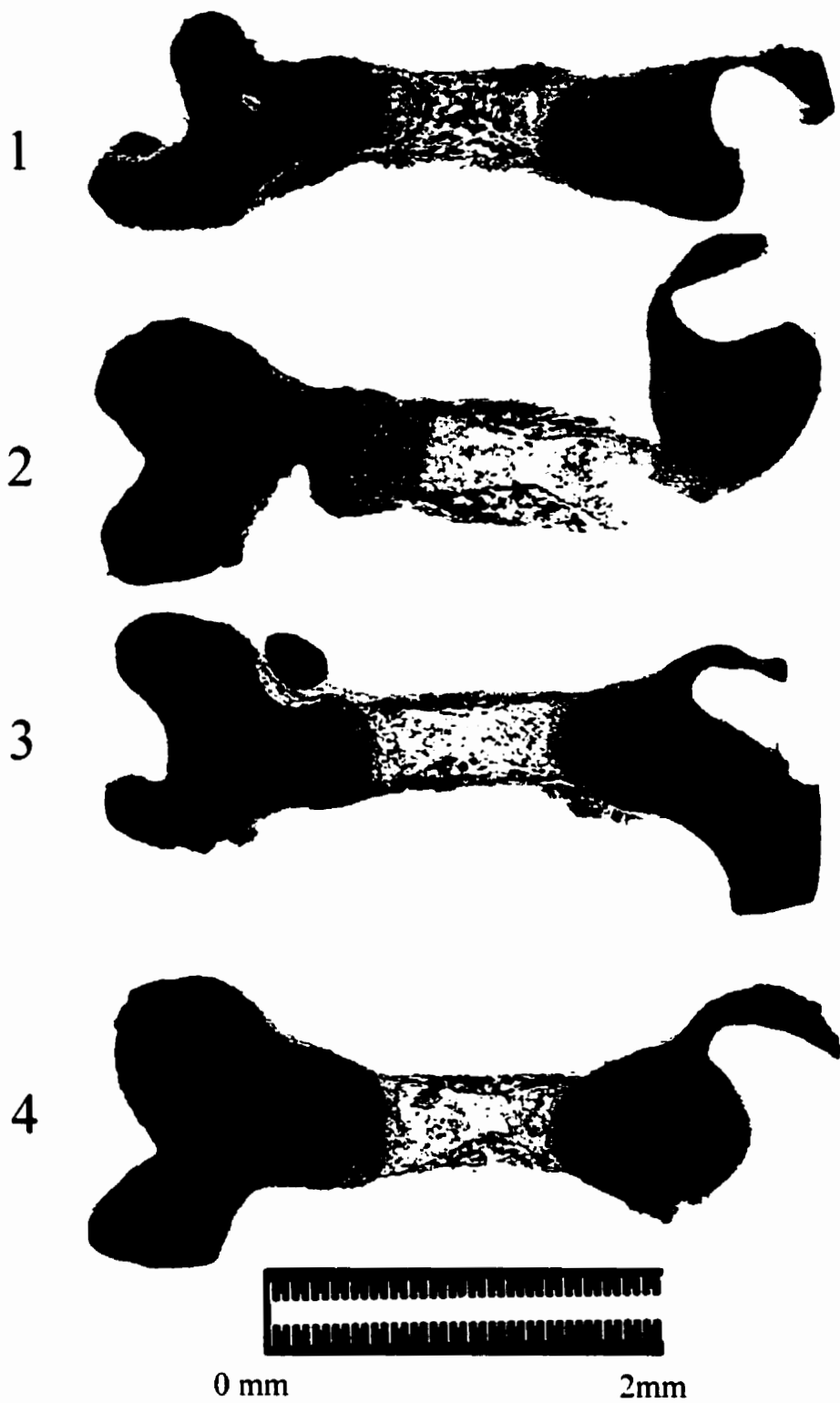
Figure 3-35: Histological sections of four different femurs after 8 days in culture with CNP at 10^{-7} M. Sections are stained in toluidene blue.
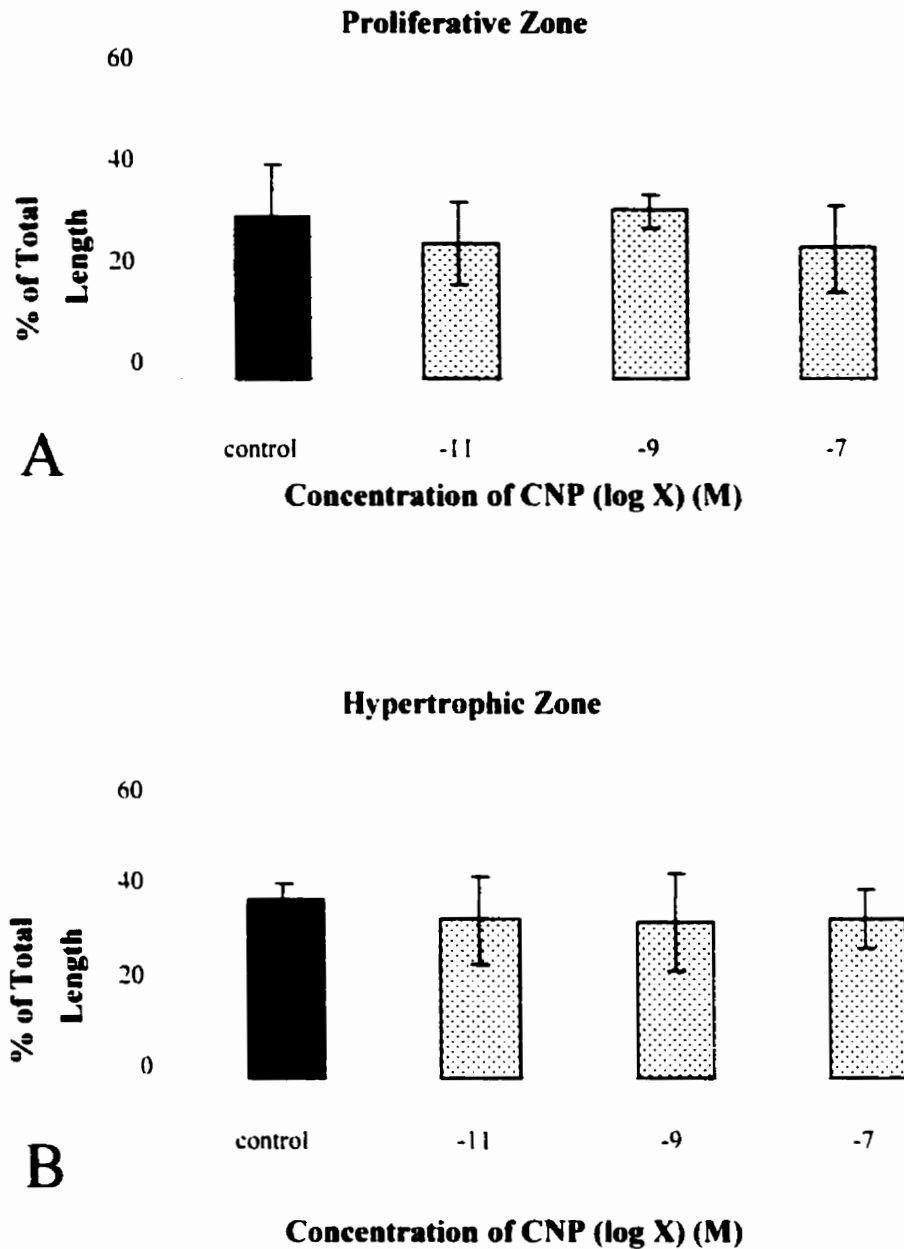
Figure 3-36: Measurements of (A) proliferative and (B) hypertrophic zones of histological sections of proximal femurs grown in culture for 8 days without CNP (n=4) and with CNP at 10^{-11} (n=3), 10^{-9} (n=4), and 10^{-7} (n=4). There is no significant difference among groups. Values are mean ± standard deviation.

Proliferative Zone

A

Concentration of CNP (log X) (M)

Hypertrophic Zone

B

Concentration of CNP (log X) (M)
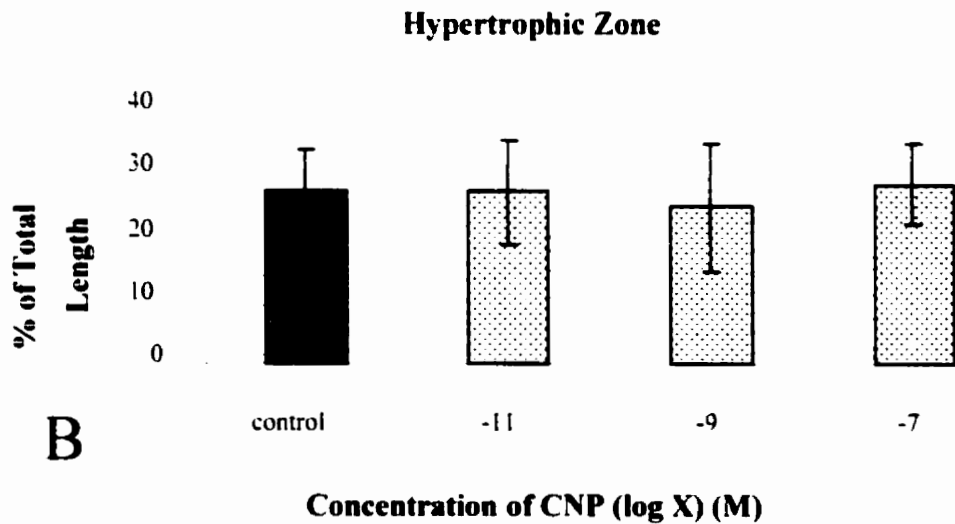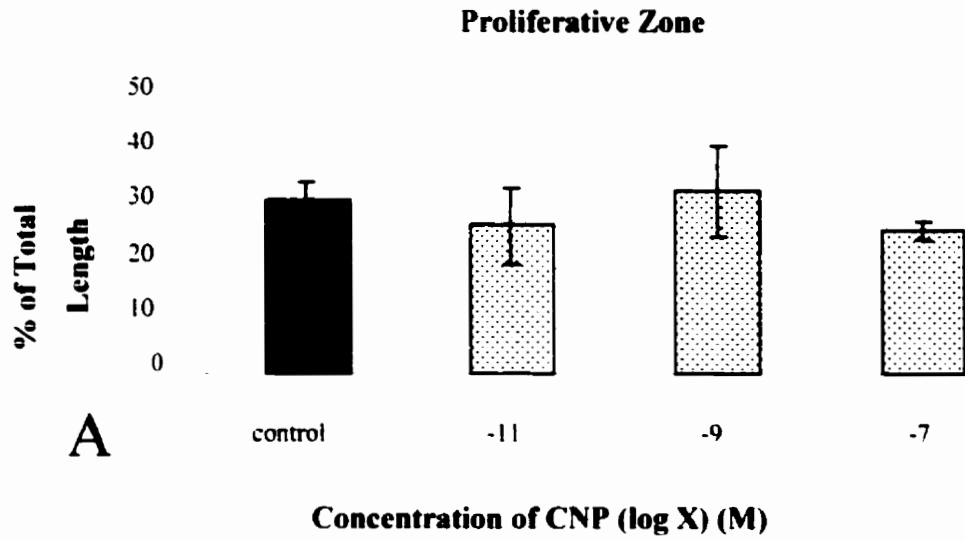
Figure 3-37: Measurements of (A) proliferative and (B) hypertrophic zones of histological sections of distal femurs grown in culture for 8 days without CNP (n=4) and with CNP at 10^{-11} (n=3), 10^{-9} (n=4), and 10^{-7} (n=4). There is no significant difference among groups. Values are mean $\pm$ standard deviation.

Morphological Analysis

Examination of the sections at 40X magnification revealed no pronounced change in the histological characteristics of the bones with the addition of CNP. The perichondrium of the bone in all experimental conditions all had rounded chondrocytes becoming a layer of three to four rows of darkly staining, flattened, elongated cells towards the outside of the bone (Figure 3-38). This definitive outer layer forms the perichondrium. Adjacent to the perichondrium is the resting zone which, at all doses, maintained a hyaline cartilage appearance with rounded chondrocytes. The proliferative zone (Figure 3-39), defined by the columns of stacked cells longitudinal to the bone axis, was less dense in the bones cultured with 10^{-9} and 10^{-7} M CNP. The hypertrophic zones in all the bones consisted of large chondrocytes with large cytoplasmic areas (Figure 3-40). The trabecular areas of all the bones had spicule formation with many cells, both osteoblasts and osteoclasts, surrounding the bony spicules (Figure 3-41).
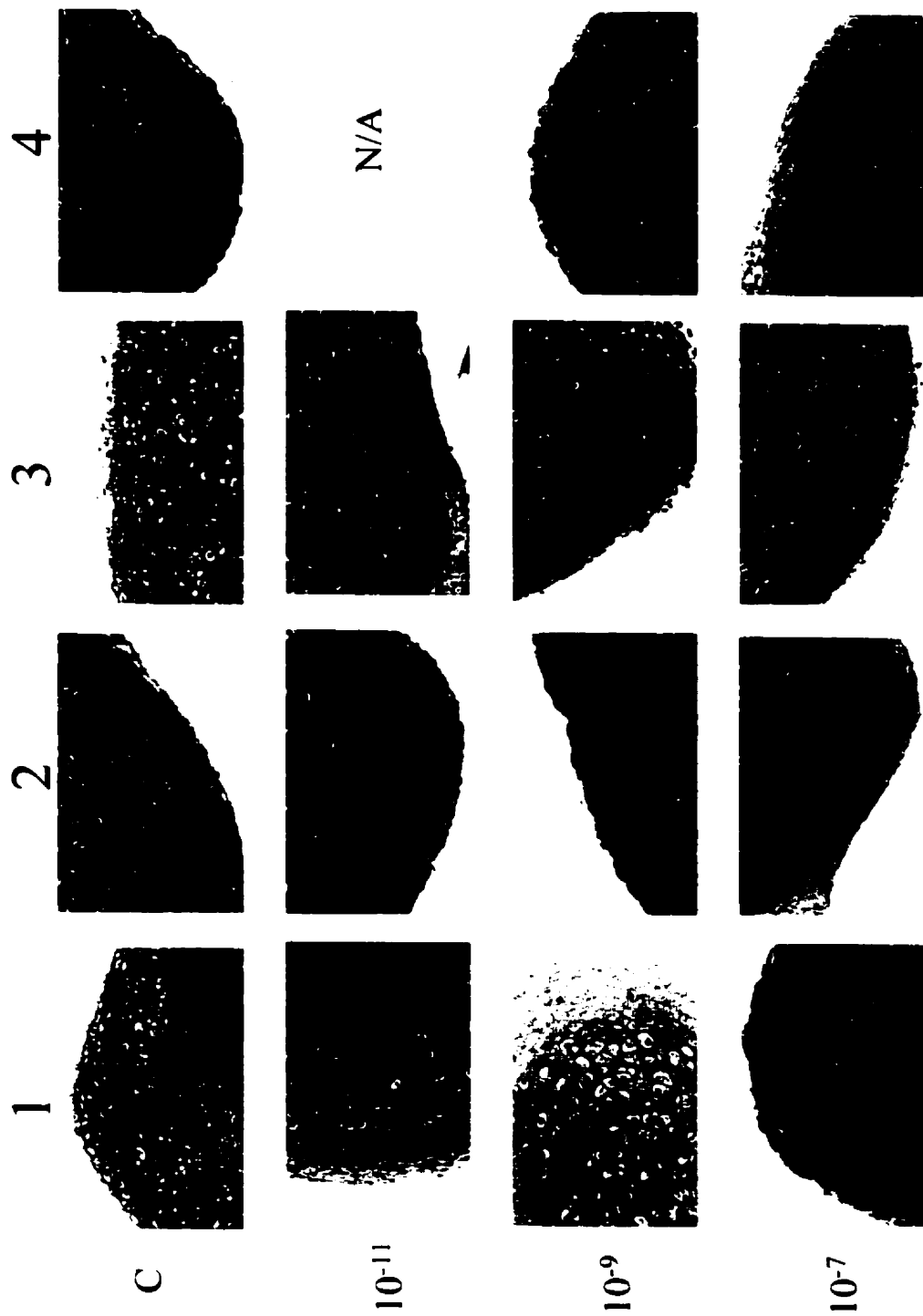
Figure 3-38: Histological sections of perichondrium of femurs grown in culture for 8 days without CNP and with CNP at 10^{-11}, 10^{-9}, and 10^{-7}. Sections are stained in toluidene blue. 40X magnification.
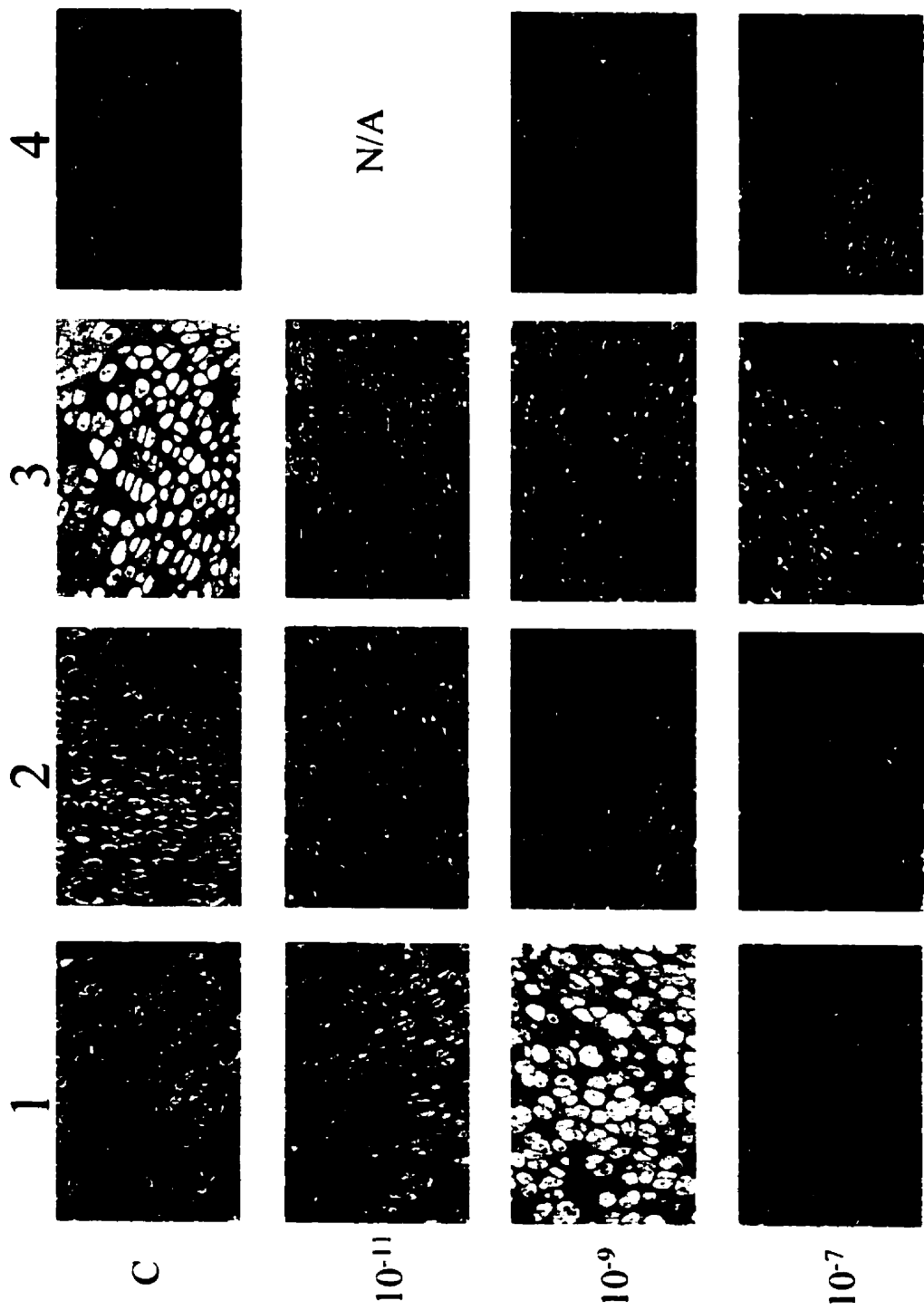
Figure 3-39: Histological sections of proliferative zones of femurs grown in culture for 8 days without CNP and with CNP at 10^{-11}, 10^{-9}, and 10^{-7}. Sections are stained in toluidene blue. 40X magnification.
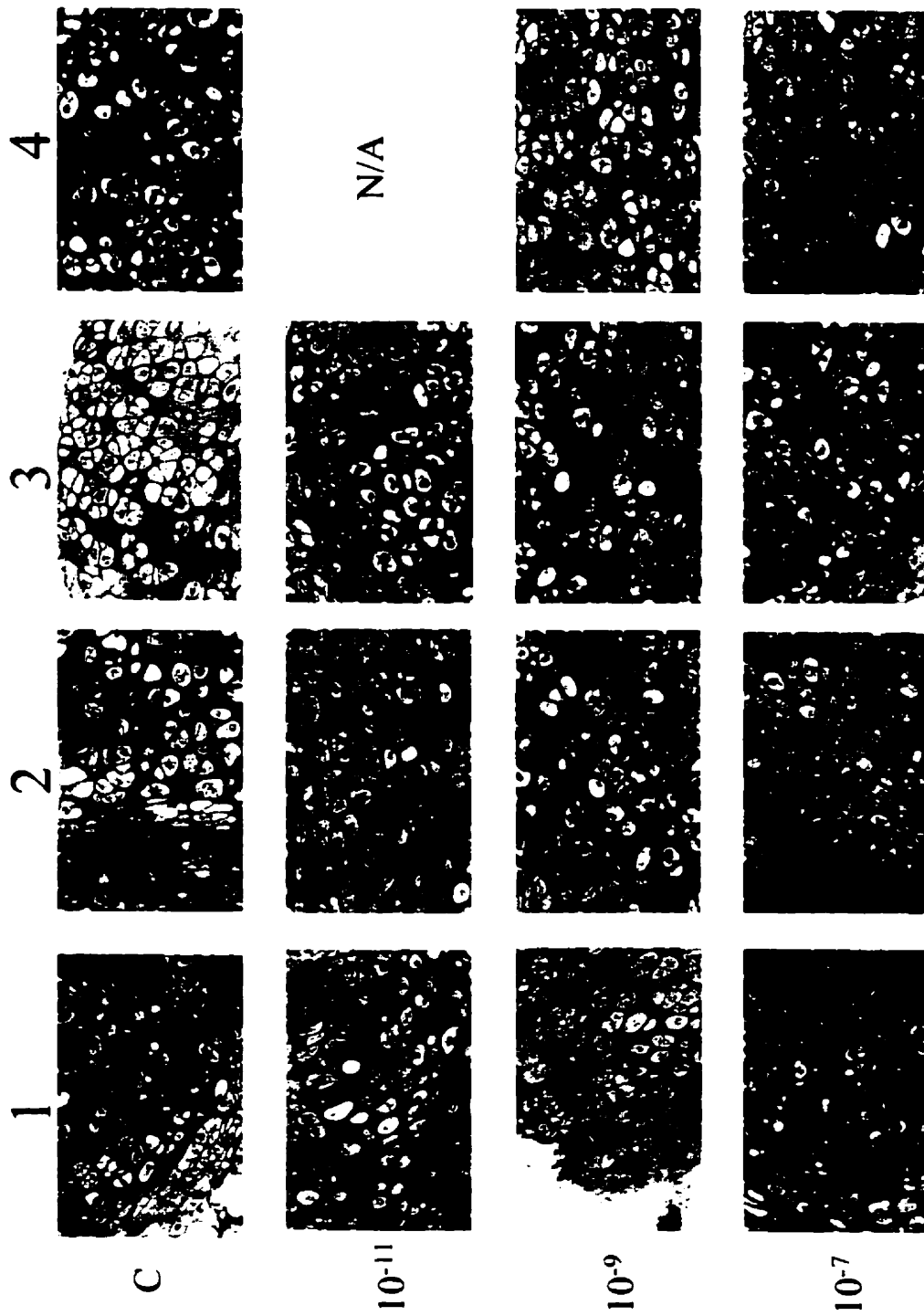
Figure 3-40: Histological sections of hypertrophic zones of femurs grown in culture for 8 days without CNP and with CNP at 10^{-11}, 10^{-9}, and 10^{-7}. Sections are stained in toluidene blue. 40X magnification.

N/A

4   3   2   1

C 10^{-11} 10^{-9} 10^{-7}
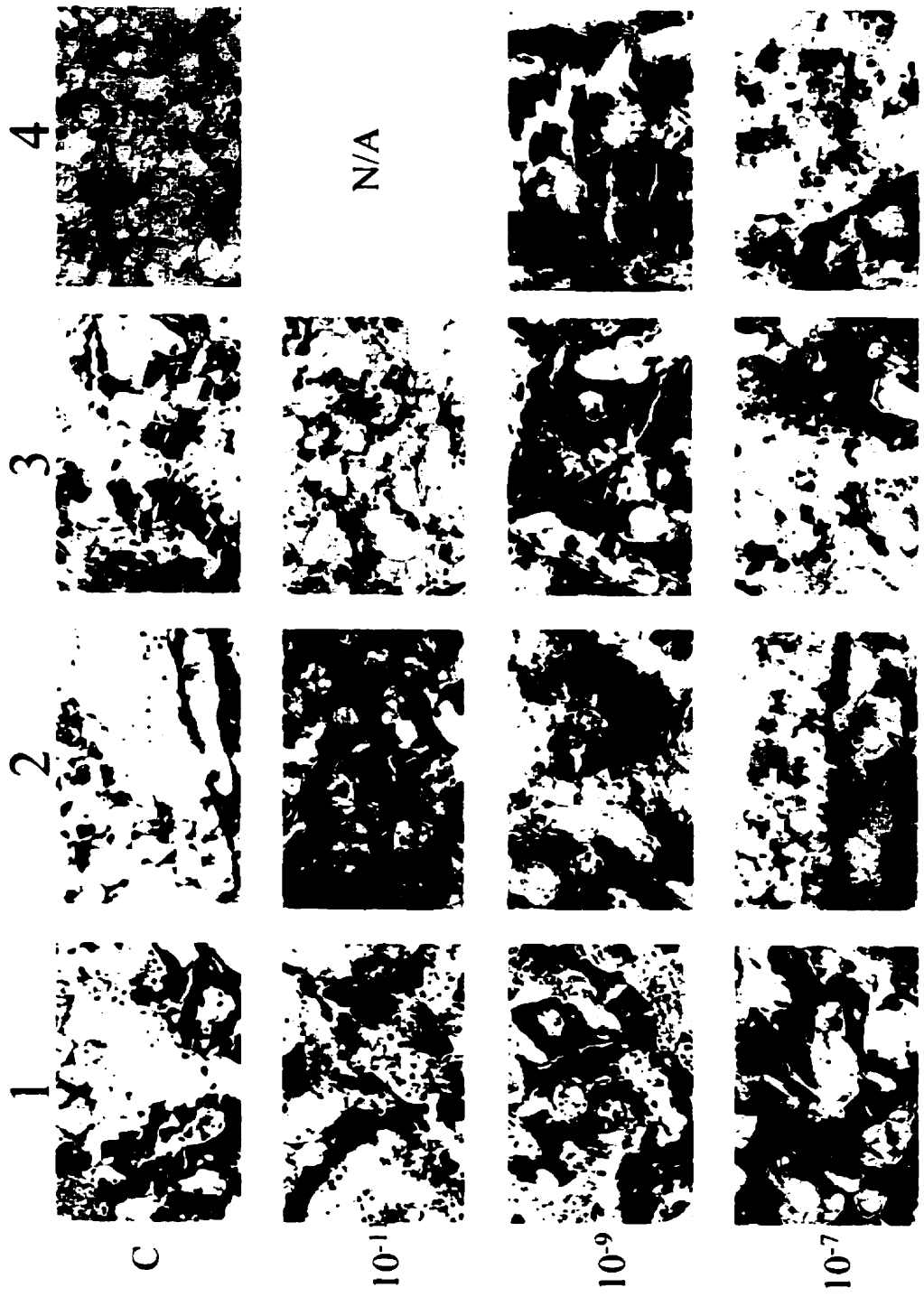
Figure 3-41: Histological sections of trabeculae of femurs grown in culture for 8 days without CNP and with CNP at 10^{-11}, 10^{-9}, and 10^{-7}. Sections are stained in toluidene blue. 40X magnification.

Chapter 4

Discussion

4.1 DISCUSSION

The mechanisms of bone formation and development have yet to be fully explained. The understanding of bone pathophysiology has many implications, including potential cures for conditions such as osteoporosis and skeletal dysplasias. Although estrogen has already been established as an important component, many other hormones may be involved in bone growth and development. One such family of hormones is the natriuretic peptides. There have been recent developments regarding the relationship between natriuretic peptides and bone development, specifically CNP.

There are many challenges in elucidating the mechanism of action of development and maintenance of bone. It is important to understand the mechanism as an entire organ as well as at the molecular level. This study investigated the actions of estradiol and CNP in *ex vivo* organ culture studies, as well as at a cellular level in *in vitro* cultured osteoblasts studies.

4.1.1 Cell culture

Cell culture of the SaOS-2 cell line was used to determine the molecular mechanisms of both estradiol and CNP on osteoblasts. Osteoblasts are responsible for producing growth factors and synthesizing and secreting matrix proteins that are incorporated into the skeleton which are important in bone growth and development. This particular cell line was used because it was a human cell line that had already been characterized and used in various studies, allowing for results to be extrapolated to human conditions. The results from culture of an immortal cell line such as SaOS-2, allow for reproduction of results since the phenotype of the cell should not change.

Cell Proliferation

In the present investigation, estradiol at concentrations of 10^{-13} and 10^{-9} M caused an increase of ^{3}H-thymidine uptake 36 % and 33 % respectively from control conditions. However, estradiol had no significant effect on cell proliferation using the CellTiter 96® Aqueous One Solution. In a human immortalized fetal cell line (hFOB/ER9) which contains a high level of estrogen receptor, estradiol at concentrations between 10^{-11} and 10^{-8} M caused a dose dependent decrease in cell proliferation (Robinson et al., 1997). In a study by Fohr et al. (2000), estradiol at 10^{-8} M significantly increased cell proliferation in SaOS-2 cells, but estradiol at 10^{-9} M had no significant effect. However, estradiol at both doses significantly increased cell proliferation in MG 63 (14 year old male) and TE 85 (13 year old female) cell lines but did not induce proliferation in the HOS 58 cell line (21 year old male). These conflicting results from previous studies and the present study may be due to the different cell lines used, the different concentrations of estradiol administered, and the various media supplementation.

CNP had a no significant influence on cell proliferation from control using either ^{3}H-thymidine incorporation or the CellTiter 96® Aqueous One Solution. In a study by Hagiwara et al. (1996), CNP has been shown to decrease cell proliferation by almost 40% in primary culture of osteoblast-like cells from rat calvariae. It has also been shown to dose-dependently suppress cell proliferation by 10^{-9} M concentrations and higher in the mouse osteoblastic cell line, MC3T3-E1 (Suda et al. 1996). These are in disagreement with the results shown in this study as no significant effect was seen after administration of CNP. However, studies with CNP with SaOS-2 cells have not been previously investigated. The outcome may be specific to the cell line.

Alkaline Phosphatase

Estradiol caused a significant increase in alkaline phosphatase activity at 10^{-15} M and a significant decrease at 10^{-9} M in the present investigation. Alkaline phosphatase activity was increased in SaOS-2 cells in a study conducted by Fohr et al. (2000) at an estradiol concentration of 10^{-8} M, but had no significant change in activity at 10^{-9} M. Estradiol was also seen to induce alkaline phosphatase activity in the human osteoblast cell line hFOB/ER9 at concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M (Robinson et al. 1997). The increase and decrease in ALP activity at different concentrations is not a trend that has not been demonstrated in previous studies. The results of the present study conflict previous results in that the high concentrations of estradiol decrease ALP activity, whereas the lower concentrations increase ALP activity.

In the present study, alkaline phosphatase activity was significantly increased with cells cultured with 10^{-11} M of CNP. ALP activity, in a study by Suda et al. (1996) was not significantly affected in MC3T3-E1 cells at concentrations of CNP between 10^{-9} and 10^{-5} M. However, a study by Hagiwara et al. (1996) shows that CNP at 10^{-7} M in primary culture of osteoblast-like cells from rat calvariae caused an increase in ALP activity of approximately 140% from basal levels. The results of this study are in accordance with the study by Suda et al. (1996) and in conflict with the Hagiwara et al. study (1996). The increase seen in ALP activity using the osteoblast-like cells from rat calvariae may be a result of a different cell phenotype from the SaOS-2 cells. Studies have not shown treatment with CNP at 10^{-11} M.

There were many limitations in using the SaOS-2 cell line. Cell lines vary in their receptor population and function when isolated and cultured. Therefore it is difficult to

conclude that the effects observed in this study can be used to describe the exact mechanism of action with CNP and estradiol. Further studies with other human osteoblastic cell lines must be done to show consistency among cell lines. Bone formation, growth, and development involves various cellular components other than osteoblasts, such as osteoclasts and osteocytes. By isolating the osteoblasts, the interactions between cells can not be understood. Furthermore, actions of hormones such as CNP and estradiol may be different when these other cells are introduced into the system.

4.1.2 Organ Culture

Organ culture is a good model for studying a complex system such as bone. In previous studies, it has been able to provide valuable information about physiology, pathology, and bone development (Stern et al., 1979). Furthermore, Sosklone (1986) has demonstrated that organ culture with fetal mice long bones has been shown to be a suitable model for the study of bone modelling, measuring bone growth, formation, calcification, and resorption. It incorporates all factors in bone as they would be interacting *in vivo* allowing for different cell types and factors to exert their effects on one another.

Establishment of Organ Culture Model

In the present investigation, both femurs and tibiae demonstrated a significant increase in growth in both length and area in proximal, distal, and total bone. An increase in both trabecular area and length however was not seen. Soskolne et al. (1985) showed that in radii and ulnae of mice from the Sabra strain did have a significant

increase in total and trabecular length over the course of 6 days. Although trabecular length did increase in these bones, the media was supplemented with FCS. The most rapid phase of growth occurred during the first two days in the study conducted by Sosklone and similar results are found in this study. The major increase in growth is usually found by day 4 with a slowing in growth by day 8.

Effect of Estradiol on Bone Growth

There were no significant effects in the present study of estradiol on the culture of tibiae or femurs except at 10^{-13} M where an increase in total length was observed at day 4. Estradiols are known to exert a more complex action on osseous tissue than on the cartilaginous tissue found in fetal mouse bones (Liskova, 1976). In previous studies, estradiol has been shown to have negligible effects on fetal mouse long bones in culture (Canalis et al., 1978). This is similar to the results of this study. However, an increase in bone length has been observed in sex-specific studies (Schwartz et al., 1991). Bones from female fetuses have shown a significant increase in both trabecular and total bone length. This may explain why significance was reached in certain concentrations and not others if the bones at 10^{-13}M were from female fetal mice.

Effect of CNP on Bone Growth

In the present experiment, both areas and lengths of fetal mouse femurs and tibiae are being cultured with CNP at concentrations of 10^{-11}, 10^{-9}, and 10^{-7} M. At day 4 and day 8, a significant increase in proximal, distal and total areas in the tibia was seen between control and CNP at 10^{-7} M. Similar results were seen with culture of femurs, however no significant increases were seen at day 4 in any of the doses or any areas. It has previously been established that CNP affects bone growth using different species,

measurements. and doses of CNP. In a study by Suda et al. (1998), a 60% increase in total bone length from control was seen in embryonic mouse tibiae cultured with CNP at 10^{-6} M. Furthermore. Mericq et al. (2001) recently showed a dose dependent increase in growth rate at 10^{-8}. 10^{-7}. and 10^{-6} M concentrations of CNP in fetal rat metatarsals. with a maximum growth at 10^{-6} M. CNP has a significant increase in both length and area only at a concentration of 10^{-7} M and not at any of the lower concentrations investigated. This is in agreement to the study by Mericq et al. (2001) where an increase in growth rate was seen at doses of 10^{-8} M and higher. The 30-50% increase in length and area at 10^{-7} M is comparable to the 60% increase in length with 10^{-6} M in the study conducted by Suda et al (1998).

Morphological Studies

No significant changes in either morphology or in relative zones of the bones may indicate that CNP does not exert its effects on a localised population of cells. Because growth seems to be uniform through all zones examined. CNP may exert a non-specific effect on the long bone. A study by Mericq et al. (2000) determined the height of the terminal hypertrophic cells and the number of hypertrophic cells per section to be increased with the addition of CNP at 10^{-9} and 10^{-6} M concentrations.

Organ culture using mouse femurs and tibiae have limitations in both its experimental design and its application. It is difficult to dissect intact femurs and tibiae from the mouse embryo due to their small sizes and their cartilaginous structure. If improperly dissected the bones can become damaged or broken which may affect the results. Extrapolating studies on mice to humans is also a challenge that faces this model. Although there have been documented similarities between mouse and human,

they are not the same species. The effects seen in the mouse organ culture may not be relevant to humans if estradiol and CNP interact differently. Finally. the model. although using intact bone. is still not an *in vivo* model. Interactions with other systems. factors. and hormones in the body may have a profound effect on the interactions of these hormones with bone growth and development.

4.1.3 General Discussion

CNP and estradiol were administered to cultured osteoblasts to elucidate the role of the osteoblast in bone development. Estradiol had an effect in the cell proliferation measured by ^{3}H-thymidine incorporation at 10^{-9} and 10^{-13} M concentrations. It however did not demonstrate an increase in cell proliferation measured through metabolic activity. The ALP activity. a biochemical marker for osteoblast cell function. increased at 10^{-15} M and decreased at 10^{-9} M. This suggests that estradiol may have actions on the cell dependent on concentration. The different actions exhibited by estradiol may provide an explanation to the conflicting results of cell proliferation in this study compared to previous studies. CNP did not have any effect on cell proliferation and only had increased ALP activity at 10^{-15} M. These results suggest that CNP does not play a major role in mature osteoblasts.

The results from the organ culture studies. however. illustrate a different picture from those from the *in vitro* studies. In the intact bone. the effects of estradiol were negligible. although an increase in length at day 4 was evident with a dose of 10^{-13} M. The only significant increase in trabecular length in all experiments was also seen at this dose. The effect of estradiol at a low dose may indicate the sensitivity of the estradiol

receptors in the bone. At higher doses. the effect may be diminished by overactivation of receptors. The sex specific increase in total bone length as shown in the study by Schwarz et al. (1991) may also have played a role. however sex of the fetuses was not determined at the time of dissection in this study. These results suggest that estradiol may not exert a major effect on pre-natal development. CNP. however. had significant effects in increasing area at doses of 10^{-7} M but not length. This does not agree with previous studies that show an increase in length. However. these studies did not investigate the increase in area. The increase in both tibiae and femurs were significant at day 8 of culture and also at day 4 in tibiae. The tibiae are smaller than the femurs and this size difference may account for the increase in area shown at day 4. CNP may have been able to enter the tibiae more freely due to the decreased size and direct its actions upon the appropriate cells faster than in the femurs. The increase in area and not in length also provides a mechanism of action of CNP. It may act to increase the thickness of the bone but not involved in bone length. These results suggest that CNP may be an important hormone in the regulation of fetal skeletal development.

4.2 FUTURE DIRECTIONS

The present investigation is only the beginning of discovering the potential impact of natriuretic peptides. specifically CNP. on bone formation and development. The following investigations could be performed in order to determine the exact role of CNP in regards to the pathophysiology of bone.

1. Through histological staining. determine the number of osteoblasts and osteoclasts per unit of bony spicule in organ culture histological sections. This

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can be done by staining for alkaline phosphatase and acid phosphatase indicating osteoblasts and osteoclasts respectively.

2. Determine level of GC-A, GC-B and C receptors in both organ culture of femurs and tibiae as well as in cell culture of SaOS-2 cells.

3. Autoradiography can be performed in order to determine and localize the activity of cells and binding of CNP. To determine activity, incubate femurs and tibiae with ^{3}H-thymidine 12 hours before termination of experiment. Fix, section and mount the bones and expose them. For the binding study, incubate femurs and tibiae with labeled CNP.

4. Determine the effect of all natriuretic peptides (ANP, BNP, and CNP) on both organ culture and cell culture to see if there are any differences.

5. *In vivo* experiments with ovariectomized and normal mice. Ovariectomized mice may exhibit osteoporotic conditions, thus providing an excellent model for osteoporosis. Mice would then be subject to treatments of CNP, estradiol or a combination of the two. Morphological studies on the bones of these treated mice could then follow to determine the effects of these hormones in the body. This experiment could also be done on pregnant females to determine the effect on the mother as well as the fetuses.

6. A previous study has shown that CNP may be linked to estradiol. In the female reproductive organs, the highest amount of CNP transcripts and immunoreactive transcripts in ovary and uterus (Huang et al., 1996) which are also areas with high concentrations of estradiol. It has been shown that CNP expression in the uterus varies during the estrus cycle with maximal expression at proestrus (Acuff et al.,

1997) and that estradiol induces CNP gene expression in mouse uterus (Acuff et al.. 1997). Determining basal levels of CNP mRNA in both cell culture and organ culture and investigating the effect of estradiol on these levels may provide an interesting relationship between the two hormones.

4.3 SUMMARY AND CONCLUSIONS

The findings of this study indicate that estradiol primarily affects the function of mature osteoblasts as opposed to the cartilaginous structures found in fetal bones. The role of estradiol has been shown to be vital in mature bone as loss of endogenous estradiol through menopause leads to osteoporosis. The effect may not be as crucial in the developing bones. However. CNP seems to have the opposite effects. It demonstrates a crucial role in the development of fetal bones not only in this study but in previous investigations as well. The overstimulation of CNP due to a lack of the clearance receptor (NPRC) caused increased skeletal growth (Matsukawa et al. 1999) and the lack of CNP caused dwarfism in mice (Chusho et al.. 2001).

The importance of these two hormones in bone physiology remains to be fully elucidated. However this study has provided insight as to the time period that they may be crucial for bone development and/or maintenance. Further studies must be done in order to fully understand the mechanism of action of these two hormones in both *ex vivo* and *in vitro* experiments.

References

REFERENCES

Acuff CG. Huang H. Steinhelper ME. **Estradiol induces C-type natriuretic peptide gene expression in mouse uterus.** *Am J Physiol.* 273(6): H2672-H2677. 1997.

Anand-Srivastava MB. Sairam MR. Cantin M **Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system.** *J. Biol. Chem.* 265: 8566-8572. 1990.

Anand-Srivastava MB. Sehl PD. Lowe DG **Cytoplasmic domain of natriuretic peptide receptor-C inhibits adenylate cyclase.** *J. Biol. Chem.* 271: 19324-19329. 1996.

Arts J. Kuiper GG. Janssen JM. Gustafsson JA. Lowik CW. Pols HA. van Leeuwen JP. **Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells.** *Endocrinology.* 138: 5067-5070. 1997.

Ashton BA. Allen TD. Howlett CR. Eaglesom CC. Hattori A. Owen ME. **Formation of bone and cartilage by marrow stromal cells and in diffusion chambers *in vivo*.** *Clin Orthop Relat Res.* 151: 294-307. 1981.

Bellido T. Girasole G. Passeri G. Yu XP. Mocharla H. Jilka RL. Notides A. Manolagas SC. **Demonstration of estrogen and vitamin D receptors in bone marrow-derived stromal cells: up-regulation of the estrogen receptor by 1,25-dihydroxyvitamin-D₃.** *Endocrinology.* 133: 553-562. 1993.

Beresford J. Gallagher JA. Poser JW. Russell RGG. Production of osteocalcin by human bone cells in vitro. **Effects of 1,25-(OH)₂ D3, 24,25-(OH)₂ D3, parathyroid hormone, and glucocorticoids.** *Metabolic Bone Disease and Related Research* 5: 229-234: 1984.

Berridge MV. Tan AS. **Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction.** *Arch Biochem Biophys.* 303: 474-82. 1993.

Bonewald LF. Kester MB. Schwartz Z. et al. **Effects of combining transforming growth factor and 1,25 dihydroxyvitamin D3 on differentiation of human osteosarcoma (MG-63).** *Journal of Biological Chemistry* 267: 8943-8949: 1992.

Burkitt HG. Young B. Heath JW. "Skeletal Tissues" in Wheater's Functional Histology. (Churchill Livingstone. London). pp. 170-190. 1993.

Burnett Jr. JC. Cranger JP. Opgenorth TJ. **Effects of systemic atrial natriuretic factor on renal function and renin release.** *Am. J. Physiol. (Renal Fluid Electrolyte Physiol. 16)*: F836-F866. 1984.

Cahill PA. Hassid A. **ANF-C-receptor-mediated inhibition of aortic smooth muscle cell proliferation and thymidine kinase activity.** *Am. J. Physiol. (Renal Integrative Comp. Physiol. 35)*: R194-R203. 1994.

Canalis E. Raisz LG. **Effect of sex steroids on bone collagen synthesis in vitro.** *Calcif Tissue Res.* 25:105-10. 1978.

Chinkers M. Garbers DL. **Signal transduction by guanylyl cyclases.** *Annu. Rev. Biochem.* 60. 553-575. 1991.

Chomczynski P. Sacchi N. **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.** *Anal Biochem.* 162:156-9. 1987.

Chusho H. Tamura N. Ogawa Y. Yasoda A. Suda M et al. **Dwarfism and early death in mice lacking C-type natriuretic peptide.** *Proc Nat Acad Sci.* 98: 4016-4021. 2001.

Consensus Development Conference. **Prophylaxis and treatment of osteoporosis.** *Am J Med.* 94: 646-650. 1993.

Dagnino L. Drouin J. Nemer M. **Differential expression of natriuretic peptide genes in cardiac and extracardiac tissues.** *Mol. Endocrinology.* 5: 1292-1300. 1991.

Hagiwara H. Inoue A. Yamguchi A. Yokose S. Furuya M. Tanaka S. Hirose S. **cGMP produced in response to ANP regulates proliferation and differentiation of osteoblastic cells.** *Am J Physiol.* 270(39): C1311-C1318. 1996.

Davis VL. Couse JF. Gray TK. Korach KS. **Correlation between low levels of estrogen receptors and estrogen responsiveness in two rat osteoblast-like cell lines.** *J Bone Miner Res.* 9: 983-991. 1994.

Engel A. M. Schoenfeld R. Lowe DG. **A single residue determines the distinct pharmacology of rat and human natriuretic peptide receptor-C.** *J. Biol. Chem.* 269: 17005-17008. 1994.

Eriksen EF. Colvard DS. Berg NJ. Graham ML. Mann KG. Spelsberg TC, Riggs BL. **Evidence of estrogen receptors in normal human osteoblast-like cells.** *Science.* 241: 84-86. 1988.

Farley JR. Hall SL. Tanner MA. Wergedal JE. **Specific activity of skeletal alkaline phosphatase in human osteoblast-line cells regulated by phosphate, phosphate esters, and phosphate analogs and release of alkaline phosphatase activity inversely regulated by calcium.** *J Bone Min Res* 9: 497-508: 1994.

Fedde KN. **Human osteosarcoma cells spontaneously release matrix-vesicle-like structures with the capacity to mineralize.** *Bone and Mineral* 17: 145-151: 1992.

Fohr B. Schulz A. Battmann A. **Sex steroids and bone metabolism: comparison of in vitro effects of 17beta-estradiol and testosterone on human osteosarcoma cell lines of various gender and differentiation.** *Exp Clin Endocrinol Diabetes*. 108: 414-23. 2000.

Frost HM. **The pathomechanics of osteoporosis.** *Clin Orthop* 200: 198-225. 1985.

Fuller F. Porter JG. Arfsten AE. Miler J. Schilling JW. Scarborough RM. Lewicki JA. Schenk DB. **Atrial natriuretic peptide clearance receptor.** *J Biol Chem* 19: 9395-9401. 1988.

Fujishige K. Yanaka N. Akatsuka H. Omori . **Localization of clearance receptor in rat lung and trachea: association with chondrogenic differentiation.** *Am J Physiol*. 274 (*Lung Cell Mol Physiol* 8): L425-L31. 1998

"Greenberg Center for Skeletal Dysplasias" http://www.med.jhu.edu/Greenberg.Center

Hagiwara H. Inoue A. Yamaguchi A. Yokose S. Furuya M. Tanaka S. Hirose S. **cGMP produced in response to ANP and CNP regulates proliferation and differentiation of osteoblastic cells.** *Am J Physiol*. 270 (*Cell Physol* 3): C1311-C1318. 1996.

Harris H. **The human alkaline phosphatase, what we know and what we don't know.** *Clinica Chimica Acta* 186: 133-136: 1989.

Hauschaka PV. Mavrakos AE. Iafrati MD et al.. **Growth factors in bone matrix, isolation of multiple types by affinity chromatography on heparin-sepharose.** *J Biol Chem* 261: 665-674. 1986.

Heaney RP. Recker RR. Saville PD. **Menopausal changes in bone remodelling.** *J Lab Clin Med*. 92: 964-970. 1978.

Henderson S. Sillence D. Loughlin J. Bennetts B. Sykes B. **Germline and somatic mosaicism in achondroplasia.** *J Med Genet*. 37(12): 956-8. 2000.

Hirata M. Chang C-H. Murad. **Stimulatory effects of atrial natriuretic factor on phosphoinositide hydrolysis in cultured bovine aortic smooth muscle cells.** *Biochem Biophys Acta*. 1010: 346-351. 1996.

Howell DS. Dean DD in Disorders of Bone and Mineral Metabolism. eds. Coe FL & Favus MJ (Raven. New York). pp. 313-353. 1992.
Huang H. Acuff CG. Steinhelper ME. **Isolation, mapping, and regulated expression of the gene encoding mouse C-type natriuretic peptide.** *Am J Physiol*. 271(40): H1565-H1575. 1996.

Iannotti JP **Growth plate physiology and pathology.** *Orthop Clin North Am*. 21: 1-17. 1990.

Ikegami A. Inoue S. Hosoi T. Mizuno Y. Nakamura T. Ouchi Y. Orimo H.
Immunohistochemical detection and northern blot analysis of estrogen receptor in osteoblastic cells. *J Bone Miner Res.* 8: 1103-1109. 1993.

Ikegami A. Inoue S. Hosoi T. Kaneki M. Mizuno Y. Akedo Y. Ouchi Y. Orimo H. **Cell cycle-dependent expression of estrogen receptor and effect of estrogen on proliferation of synchronized human osteoblast-like osteosarcoma cells.** *Endocrinology.* 135: 782-789. 1994.

Inoue A. Himura Y. Hirose S. Yamaguchi A. Furuya M. Tanaka S. Hagiwara H. **Stimulation by C-type natriuretic peptide of the differentiation of clonal osteoblastic MC3T3-E1 cells.** *Biochem. Biophys. Res. Commun.* 221: 703-707. 1996.

Jilka RL. **Cytokines, bone remodelling, and estrogen deficiency: a 1998 update.** *Bone.* 23: 75-81. 1998.

Jones SJ. Boyde A. Ali NN et al. **A review of bone cell substratum interactions.** *Scanning.* 7: 5-24. 1985.

Junquiera LC. Carneiro J. Kelley. RO Basic Histology. Appleton & Lange. Stamford. CN. 1995.

Katzung BG. Basic and Clinical Pharmacology 7th Edition. Appleton & Lange. Stamford.CN. 654-660. 706-709. 1998.

KleerekoperM . Villancuva AR. Stanciu J et al. **the role of three-dimensional trabecular microstructure in the pathogenesis of vertebral compression fractures.** *Calcif Tissue Int.* 37: 594-597. 1985.

Kleerekoper. M. **Biochemical markers of bone remodeling.** *Am J Med Sci.* 312(6): 271-277. 1996.

Koller KJ. Lowe DG. Bennet GL. Minamino N. Kangawa K. Matsuo H. Goeddel DV. **Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP).** *Science* 252: 120-123. 1991.

Komm BS. Terpening CM. Benz DJ. Graeme KA. Gallegos A. Korc M. Greene GL. O'Malley BW. Haussler MR. **Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells.** *Science.* 241: 81-84. 1988.

Kuiper GG. Carlsson B. Grandien K. Enmark E. Haggblad J. Nilsson S. Gustafsson JA. **Comapriosn of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta.** *Endocrinolgy.* 138: 863-870. 1997.

Kuiper GG. Enmark E. Pelto-Huikko M. Nilsson S. Gustafsson JA. **Cloning of a novel receptor expressed in rat prostate ovary.** *Proc Natl Acad Sci USA.* 93: 5925-5930. 1996.

Kurland ES. Cosman F. McMahon DJ. Rosen CJ. Lindsay R. Bilezikian JP. **Parathyroid hormone as a therapy for idiopathic osteoporosis in men: effects on bone mineral density and bone markers.** *J Clin Endocrinol Metab.* 85(9):3069-76. 2000.

Lichtler A. Stover ML. Angilly J. Kream B. Rowe DW. **Isolation and characterization of the rat alpha 1(I) collagen promoter. Regulation by 1, 25-dihydroxyvitamin D₃.** *J Biol Chem.* 264: 3072-3077. 1989.

Liskova M. **Influence of estrogens on bone resorption in organ culture.** *Calcif Tissue Res.* 22:207-18. 1976.

Maack T. Suzuki M. Almeida FA. Nussenzveig D. Scarborough RM. McEnroe GA. Lewicki JA. **Physiological role of silent receptors of atrial natriuretic factor.** *Science* 238. 675-678: 1987.

Maack T. **Role of atrial natriuretic factor in volume control.** *Kidney Int.* 49. 1732-1737. 1996.

Majeska RJ. Rodan GA. **Alkaline phosphatase inhibition by parathyroid hormone and isoproterenol in a clonal rat osteosarcoma cell line: possible mediation by cAMP.** *Calcif. Tissue Int.* 34:59-66. 1982.

Malone JD. Teitelbaum GL. Griffin GL. Senior RM. Kahn AJ. **Recruitment of osteoclast precursors by purified bone matrix constituents.** *J Cell Biol* 92: 227-238: 1982.

Manolagas SC. Jilka RL. **Bone marrow, cytokines, and bone remodelling: emerging insights into the pathophysiology of osteoporosis.** *N Eng J Med.* 332: 305-311. 1995.

March of Dimes Perinatal Data Center. 2000. March of Dimes Birth Defects Foundation

Marieb EN. "Bones and Skeletal Tissue" in Human Anatomy and Physiology Third Edition. Benjamin/Cummings Publishing Company Inc.. Redwood City. 155-177. 1995.

Matsuwaka N. Grzesik WJ. Takahashi N. Pandey KN. Pang S. Yamauchi M. Smithies O. **The natriuretic peptide clearance receptor locally modulates the physiological effects of natriuretic peptide system.** *Proc. Natl. Acad. Sci.* 96: 7403-7408: June 1999.

McKenna MJ. Frame B. **Hormonal influences on osteoporosis.** *Am J Med.* 82(1B):61-7. 1987.

McSheehy PMJ, Chambers TJ. **Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone.** *Endocrinology.* 118: 824-828, 1986.

Mericq V, Uyeda JA, Barnes KM, De Luca F, Baron **Regulation of fetal rat bone growth by C-type natriuretic peptide and cGMP** *J.Pediatr Res.* 47(2):189-93, 2000.

Millet I, McCarthy TL, Vignery A. **Regulation of interleukin-6 production by prostaglandin E2 in fetal rat osteoblasts: role of protein kinase A signaling pathway.** *J Bone Miner Res.* 13: 1092-1100, 1998.

Mosselman S, Polman J, Dijkema R. **ER beta: identification and characterization of a novel human estrogen receptor.** *FEBS Lett.* 392: 49-53, 1996.

Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasai M, Obata K, Yasue H. et al., **Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide.** *J Clin Invest.* 87, 1402-1412, 1991.

Mundy GR, Luben RA, Raisz LG et al. **Bone resorbing activity in supernatants from lymphoid cell lines.** *N Engl J Med.* 290: 867-871, 1974a.

Mundy GR, Raisz LG, Cooper RA et al. **Evidence for the secretion of an osteoclast stimulating factor in myeloma.** *N Engl J Med.* 291: 1041-1046, 1974b.

Mundy GR. Bone Remodelling and Its Disorders. Martin Dunitz, London: 1999.

Nakao K, Ogawa Y, Suga S, Imura H. **Molecular biology and biochemistry of the natriuretic peptide system. I: Natriuretic peptides.** *J Hypertens.* 10, 907-912, 1992.

Nakao K, Ogawa Y, Suga S, Imura H. **Molecular biology and biochemistry of the natriuretic peptide system. II: Natriuretic peptide receptors.** *J Hypertens.* 10, 1111-1114, 1992.

Nasu M, Sugimoto T, Kaji H, Chihara K. **Estrogen modulates osteoblast proliferation and function regulated by parathyroid hormone in osteoblastic SaOS-2 cells: role of insulin-like growth factor (IGF)-I and IGF-binding protein-5.** *J Endocrinol.* 167(2):305-313, 2000.

Oldberg A, Jirskog-Hed B, Axelsson S, Heinegard D. **Regulation of bone sialoprotein mRNA by steroid hormones.** *J Cell Biol.* 109: 3183-3186, 1989.

Ogawa Y, Itoh H, Tamura N, Suga S, Yoshimasa T, Uehira M, Matsuda S, Shiona S, Nishimoto H, Nakao K. **Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene.** *J. Clin. Invest.* 93, 1911-1921, 1994.

Ogawa Y. Nakao K in Hypertension: Pathophysiology, Diagnosis, and Management, eds. Laragh JH & Brenner BM (Raven, New York), pp. 833-840, 1995.

Onoe Y. Miyaura C. Ohta H. Nozawa S. Suda T. **Expression of estrogen receptor beta in rat bone.** *Endocrinology.* 138: 4509-4512, 1997.

"Osteoporosis Society of Canada" at www.osteoporosis.ca

Oursler MJ. Osdoby P. Pyfferoen J. Riggs BL **Avian osteoclasts as estrogen target cells.** *Proc. Natl. Acad. Sci. USA.* 88: 6613-6617. 1991.

Owen M. **Lineage of osteogenic cells and their relationship to the stromal system.** *In: Peck WA. ed. Bone and Mineral Research. Annual 3:* 1-26. 1985.

Owen TA. Aronow MS. Barone LM. Bettencourt B. Stein GS. Lian JB. **Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures.** *Endocrinology.* 128: 1469-1504. 1991.

Pacifici R **Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis.** *J Bone Miner Res* 11: 1043-1051. 1996.

Paech K. Webb P. Kuiper GG. Nilsson S. Gustafsson J. Kushner PJ. Scanlan TS. **Differential ligand activation of estrogen receptors ER-alpha and ER-beta at AP1 sites.** *Science.* 277: 1508-10. 1997.

Parfitt AM. Mathews CH. Villanueva AR. Kleerekoper M. Frame B. Rao DS. **Relationships between surface, volume, and thickness of iliac trabecular bone in ageing and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss.** *J Clin Invest.* 72: 1396-409.1983.

Pfeifer A. Aszodi A. Seidler U. Ruth P. Hofmann F. Fassler R. **Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II.** *Science* 274: 2082-2086. 1996.

Porter JG. Arfsten A. Fuller F. Miller JA. Gregory LC. Lewicki. **Isolation and functional expression of the human atrial natriuretic peptide clearance receptor cDNA.** *Biochem. Biophys. Res. Commun.* 171: 796-803. 1990.

Price PA. **Vitamin K-dependent formation of bone Gla protein (osteocalcin) and its function.** *Vitamins and Hormones* 42: 65-108: 1985.

Price PA. Baukol SA. **1,25 dihydroxyvitamin D3 increases the synthesis of the vitamin K-dependent protein by osteosarcoma cells.** *Journal of Biological Chemistry* 255: 11660-11663; 1980.

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Prins BA. Weber MJ. Hu R-M. Pedram A. Daniels M. Levin ER. **Atrial natriuretic peptide inhibits mitogen-activated protein kinase through the clearance receptor.** *J. Biol. Chem* 271:4156-14162. 1996

Raisz LG. Kream BE. **Regulation of bone formation.** *N Eng J Med.* 309: 29-35. 83-89. 1983.

Recker RR in **Disorders of Bone and Mineral Metabolism** eds. Coe FL & Favus MJ (Raven. New York): 219-240. 1992.

Risteli L. Risteli J. **Biochemical markers of bone metabolism.** *Annals of Medicine* 25: 385-393. 1993.

Robinson JA. Harris SA. Riggs BL. Spelsberg TC. **Estrogen regulation of human osteoblastic cell proliferation and differentiation.** *Endocrinology.* 138: 2919-27. 1997.

Rodan GA. Martin TJ. **Role of osteoblasts in hormonal control of bone resorption – a hypothesis.** *Calcif Tissue Int.* 33: 349-351. 1981.

Rodan GA. Rodan SB. **Expressison of the osteoblastic phenotype.** in *Advances in Bone and Mineral Research. Annual II* ed Peck WA: 244-285. 1984.

Rodan SB. Imai Y. Thiede MA. Wesolowski G. Thompson D. Bar-Shavit Z. Shull S. Mann K. Rodan GA. **Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties.** *Cancer Res.* 47: 4961-6. 1987.

Rosenzweig A. Seidman CE. **Atrial natriuretic factor and related peptide hormones.** *Annu. Rev. Biochem.* 60. 229-255. 1991.

Rugh. R. The Mouse and Its Reproductive Development. Burgess Publishing Company. Minneapolis. 1968.

Sadler TW "Skeletal System" in Langman's Medical Embryology Eighth Edition. Lippincott Williams & Wilkins. Baltimore. 161-186. 2000.

Saloman Y. Londos C. Rodbell M. **A highly sensitive adenylate cyclase assay.** *Anal. Biochem.* 58:541-548. 1974.

Sato G (ed.). Functionally differentiated cell lines. New York: Alan R. Liss. 1981

Schulz S. Singh S. Bellet RA. Singh G. Tubb DJ. Chin H. Garbers DL **The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family.** *Cell* 58: 115-1162. 1989.

Schwartz Z. Soskolne WA. Neubauer T. Goldstein M. Adi S. Ornoy A.
Direct and sex-specific enhancement of bone formation and calcification by sex steroids in fetal mice long bone in vitro (biochemical and morphometric study).
Endocrinology. 129:1167-74, 1991.

Sherwood, L in Human Physiology: From Cells to Systems. (West Publishing Company, St. Paul. MN). 1993.

Shteyer A. Gazit D. Passi-Even L. Bab I. Majeska R. Gronowicz G. Lurie A. Rodan GA.
Formation of calcifying matrix by osteosarcoma cells in diffusion chambers *in vivo.*
Calcif Tis Int. 39: 59-64, 1986.

Simmonds DJ. Kent GN. Jilka RL. Scott DM. Fallon M. Cohn DV. **Formation of bone by isolated cultured osteoblasts in Millipore diffusion chambers.** *Calcif Tis Int.* 34: 291-294, 1982.

Soskolne WA. Schwartz Z. Ornoy A. **The development of fetal mice long bones in vitro: an assay of bone modeling.** *Bone.* 7:41-8, 1986.

Spranger J. **Classification of skeletal dysplasias.** *Acta Paediatr Scand Suppl.* 377:138-42, 1991.

Steln GS. Lian JB. Owen TA. **Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation.** *FASEB Journal* 4: 3111-3123; 1990.

Stern PH. Raisz LG. **Organ Culture of Bone** *in* Skeletal Research: An Experimental Approach eds. Simmons DJ and Kunin AS.. Academic Press, New York, 22-53: 1979.

Strangeways TSP. Fell HB. *Proc R Soc London, Ser B.* 99: 340-366, 1926.

Suda M. Tanaka K. Fukushima M. Natsui K. Yasoda A. Komatsu Y. Ogawa Y. Itoh H. Nakao K. **C-type natriuretic peptide as and autocrine/paracrine regulator of osteoblast.** *Biochem Biophys Res Communs.* 223(1): 1-6, 1996.

Suda M. Ogawa Y. Tanaka K. Tamura N. Yasoda A. Takigawa T. Uehira M. Nishimoto H. Itoh H. Saito Y. Shiota K. Nakao K. **Skeletal overgrowth in transgenic mice that overexpress brain natriuretic peptide.** *Proc. Natl. Acad. Sci. USA* 95: 2337-2342, 1998.

Sudoh T. Minamino N. Kangawa K. Matsuo. **C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain.** *Biochem. Biophys. Res. Commun.* 168:863-870, 1990.

Suga S. Nakao K. Hosoda K. Mukoyama M. Ogawa Y. Shirakami G. Arai H. Saito Y. Kambayashi Y. Inouye K. **Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide.** *Endocrinology* 130. 229-239; 1992.

Sutherland MK. Hui DU. Rao LG. Wylie JN. Murray TM. **Immunohistochemical localization of the estrogen receptor in human osteoblastic SaOS-2 cells:association of receptor levels with alkaline phosphatase activity.** *Bone.* 18: 361-369.1996.

Turner RT. Riggs BL. Spelsberg TC. **Skeletal effects of estrogen.** *Endocr Rev.* 15: 275-300. 1994.

Tremble GB. Tremble A. Copeland NG. Gilbert DJ. Jenkins NA. Labrie F. Giguere V. **Cloning, chromosomal localization and functional analysis of the murine estrogen receptor beta.** *Mol Endocrinol.* 11: 353-365. 1997.

Vanderschueren D. Bouillon R. **Androgens and Bone.** *Calcif Tissue Int.* 56: 341-346. 1995.

Vidal O. Kindblom L-G. Ohlsson C. **Expression and localization of estrogen receptor-β in murine and human bone.** *J Bone Miner Res.* 14: 923-929. 1999.

Yanaka N. Akatsuka H. Kawai E. Omori K **1,25-Dihydroxyvitamin D₃ upregulates natriuretic peptide receptor-C expression in mouse osteoblasts.** *Endocrinology and Metabolism* 275: E965-E973.

Calculation of Cell Concentration for Passaging

Total live cell count must first be determined by the following calculations:

TLCC = average (live cells) x V (cells) x hemocytometer dilution factor x trypan
       blue dilution factor

where: V(cells) = volume of resuspended cell pellet
       hemocytometer dilution factor = 1×10^4
       trypan blue dilution factor = 2

Example
    TLCC = $(50)(10)(1 \times 10^4)(2)$
          = 1.0×10^7 cells/ml

By using $C_1 V_1 = C_2 V_2$, one can then determine the volume of resuspended cell solution needed to achieve the desired density.

Example
To achieve a density of 1.3×10^6 cells for a 75 cm^2 flask:

$$V_{desired} = \frac{C_{desired} \times V \text{ (resuspended cell solution)}}{C(\text{resuspended cell solution})}$$
$$= \frac{(1.3 \times 10^6 \text{ cells/ml})(10 \text{ ml})}{1.0 \times 10^7 \text{ cells}}$$
$$= 1.3 \text{ ml}$$

Therefore, to achieve a density of 1.3×10^6 cells/flask, 1.3 ml of resuspended cell solution must be added to 14 ml media in a 75 cm^2 flask.

Solutions

0.04% Trypsin (1000 mL – in water)

0.2 g	KCl
0.2 g	KH$_2$PO$_4$
1.152 g	Na$_2$HPO$_4$
8.0 g	NaCl
2.0 g	glucose
0.2 g	EDTA
0.4 g	trypsin

10X PBS (1000 mL – in water)

2 g	KCl
2.4 g	KH$_2$PO$_4$
14.4 g	Na$_2$HPO$_4$
80 g	NaCl

10 X TBE (1000 mL – in water)

108 g	Tris Base
55 g	Boric Acid
7.44 g	EDTA

20 X SSC (1000 mL – in water)

175.3 g	NaCl
88.2 g	Tri-Sodium Citrate

Trypan Blue

0.4 %	Trypan Blue
	0.9% *NaCl*