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**The Role of Glutamate Receptors in Cholesteatoma
Induced Bone Resorption**

By

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**An independent study submitted in partial
Fulfillment of the requirements for the degree of:**

Master of Science in Speech and Hearing

Emphasis in Audiology

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**Approved by:
Brian T. Faddis, Ph.D., Independent Study Supervisor**

Introduction

Cholesteatoma is a progressive epithelial lesion that results from a lack of normal migration of epidermal tissues from the tympanic membrane and adjacent ear canal. The progressive erosion by cholesteatomas into middle and inner ear structures may result in permanent hearing loss, vestibular dysfunction and more severe intracranial complications. Currently, the only treatment available for cholesteatoma is surgical eradication, which often must be repeated. Osteoclasts, the more damaging part of the disease process function to break down and resorb bone. The purpose of this study is to examine a new model of cholesteatoma induced bone resorption in mice using autologous implantation of pinna dermis to the surface of the skull. Using an autologous dermal implant will produce quantifiable localized inflammatory bone remodeling similar to that seen in cholesteatoma. The first goal of this study will be to examine the consistency of autologous implants from mouse to mouse. The second goal will be to infuse the site of the implant with glutamate receptor antagonist MK-801 (NMDAR1) to see if blocking the glutamate receptors reduces osteoclast activity.

Cholesteatomas

Cholesteatomas are epithelial tumor-like growths that arise from the tympanic membrane, typically as a result of chronic otitis media. These growths usually begin in the attic of the ear, extending into the mastoid antrum, and sometimes all the way to the mastoid tip (Katz, 1994). Cholesteatomas cause progressive destruction of the middle and inner ear. Osteoclasts, the more damaging part of the disease process, break down bony structures and resorb bony tissue. Such destruction may result in hearing loss, vestibular dysfunction, and facial paralysis, as well as lethal intracranial complications (Chole et al., 2000). Cholesteatomas produce serious

intracranial complications if they erode through the dura of the middle or posterior fossa of the skull, through the lateral sinus, or into the lateral semicircular canal. They may also produce facial paralysis if the facial nerve is eroded in the middle ear or mastoid. Dizziness and true vertigo may result if the lateral semicircular canal is involved (Katz, 1994). In addition, cholesteatomas often erode the middle ear ossicles. Hearing loss as a result of cholesteatoma can vary from slight to profound depending on the size and location of the growth. Currently, the only treatment available is surgical eradication, which often has to be repeated.

Osteoclasts

Osteoclasts are found in all normal vertebrate animals. They resorb bone in response to a need for calcium. Osteoclasts are the damaging entity of the disease process because they are responsible for breaking down bony structures and resorbing bony tissue. In cholesteatoma, osteoclast activation in the bone of the middle and inner ear likely occurs through both mechanical pressure and localized inflammation (Jung et al., 2002). Chronically inflamed tissue capable of causing bone erosion is seen in many disease states, including rheumatoid arthritis, gingivitis and particle-induced osteolysis, an inflammatory response which leads to the loosening of orthopedic implants. Similarly, chronic otitis media, with and without cholesteatoma is characterized by inflamed tissue adjacent to bone. Cytokines such as interleukin-1, interleukin-6, tumor necrosis factor (TNF) and other protein mediators such as growth factors and nonprotein mediators such as prostaglandins, neurotransmitters and nitric oxide (NO) have all been associated with the destructive force that is inflammatory bone remodeling (Jung et al., 2002).

Role of Nitric Oxide in Osteoclast Activity

Nitric oxide (NO) is a neutral free radical gas known to mediate a wide range of physiologic functions including vasodilatation, neurotransmission, and muscle contractility. The enzyme family responsible for the production of NO is the nitric oxide synthase (NOS) family. Neuronal nitric oxide synthase 1 or NOS1 was first identified in neurons. It is a calcium and calmodulin-dependent, constitutively expressed isoform that has also subsequently been localized to skeletal muscle and most recently, bone (Jung et al., 2002). Studies of nitric oxide have demonstrated a significant role for NOS1 in osteoclastic bone resorption. Further studies implicate a biphasic role of NO on bone resorption – high levels inhibit resorption while low levels stimulate resorption (Jung et al., 2002).

Glutamate Receptors

Glutamate is the most abundant amino acid in the central nervous system and the most common neurotransmitter. The majority of glutamate in the brain is involved in intermediary metabolism in both neurons and glia, while only a fraction of the total glutamate pool participates in neuronal signaling. Glutamate, stored in synaptic vesicles, is released from presynaptic terminals by membrane depolarization in a Ca^{2+} dependent fashion (McDonald et al., 1990). Neurons use glutamate receptor binding as a mechanism to activate NOS1 by sequestering NOS1 near glutamate receptors. The calcium influx which results from receptor binding can then easily be made available to activate the NOS1.

Could osteoclasts use the same mechanism? Bone tissue has been shown to possess a fine network of nerve fibers and osteoclasts are known to express glutamate receptors. The glutamate neurotransmission mechanisms used by neurons are also expressed by bone cells,

osteoblasts and osteoclasts. Recent studies have examined the role of glutamate in osteoclast activity. They have shown that the glutamate receptor antagonist, MK-801, is not toxic to osteoclasts and does not interfere with adhesion of osteoclasts to a substrate, but does impair resorption activity by preventing osteoclast sealing zone formation. Also important is the role of NO as a downstream messenger of glutamate receptor activation. This means that just as glutamate and NO neurotransmitters work in the same fashion to facilitate osteoclast growth, so must glutamate receptor antagonists and the deletion of NOS1 have similar effects on the prevention of osteoclastic bone resorption.

Goals of the Present Study

This study will examine a new model of cholesteatoma induced bone resorption in mice using autologous implantation of pinna dermis to the surface of the skull. Two goals will be assessed in this study. The first goal will be to examine the consistency of autologous implants from mouse to mouse. The second goal will be to infuse the site of the implant with the glutamate receptor antagonist MK-801 (NMDAR1) to see if blocking the glutamate receptors reduces osteoclast activity.

Chole et al. (2000) in a previous study developed a model of bone resorption in mouse calvaria using keratin particles. Their model was useful to in the investigation of bone remodeling related to cholesteatoma. Based on their findings, autologous dermal implants were placed on the surface of mouse calvaria to induce bone resorption similar to cholesteatoma for the purposes of this study. This same model used by Sudhoff et al. (2003) was found to demonstrate quantifiable localized inflammatory bone remodeling similar to that seen in cholesteatoma.

Materials and Methods

Animals

Eight male wildtype mice were used in the present study. All of the mice were given food and water *ad libitum* and their circadian rhythm patterns were preserved by a 12/12 light-dark cycle. All animals were six to eight weeks of age at the time of treatment. All procedures were conducted in accord with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act. The Institutional Animal Care and Use Committee of Washington University approved the protocol for the experimental use of these animals.

Dermal Implant Procedure

Each mouse was anesthetized by an intraperitoneal injection of a mixture of ketamine (87 mg/kg) and xylazine (13.4 mg/kg). The surgical site was shaved with electric clippers and swabbed with betadine. A 6mm biopsy punch was used to obtain a dermal implant from the pinna of each mouse. A medial sagittal scalp incision was made. The periosteum was gently scraped away to expose the underlying bone. The biopsy punch was then carefully placed directly on the exposed bone of the calvaria and tacked into place using a minimal amount of surgical cyanoacrylate glue. Alzet osmotic pumps were used to systemically deliver MK-801, a glutamate receptor antagonist. To insert the pumps, the skin across the dorsum was elevated and the pump, loaded and primed, inserted down the back from the same sagittal incision made for the dermal implant. The wound was approximated and closed with two sterile wound clips being careful not to disturb the biopsy punch. Five days following the dermal implant, the animals

were euthanized with an excess of pentobarbital (>120 mg/kg). The cranial tissue with skin biopsy well attached were then excised and placed in fixative for histological processing.

Histology

The calvarial sample was placed into a phosphate-buffered fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde. This portion of the bone was subsequently decalcified in a solution of 0.35M tetrasodium EDTA, dehydrated through a graded series of acetones, and embedded in plastic (Figure 1). One micrometer sections were obtained and counterstained with toluidine blue and basic fuchsin. The number of osteoclasts in the full section were determined and expressed as the number of osteoclasts per millimeter of bone length.

Osteoclast counts

Using a microscope, osteoclasts were identified. In order to be counted as an active osteoclast, the cell must be located directly adjacent to bone and possess at least three of the following four criteria: a ruffled border, granular cytoplasm, multiple nuclei, and the absence of lamina limitans. Cell counts were obtained from 4-6 sections for each animal. In order to avoid counting the same cell twice, tissue sections were spaced 100 μm apart (Figure 2).

Statistical Analysis

Osteoclast counts were evaluated using Student's t-test for unpaired samples. In order to be statistically significant, $p < 0.050$. Means and standard deviations were plotted on a histogram. (Figure 3)

Results

Implantation of the autologous dermal biopsy punch yielded an osteoclastic response in calvarial bone that was similar to results of the Chole et al. (2000) keratin implant study, implying that this method will be useful for further investigations of localized inflammatory bone resorption related to cholesteatoma. Local infusion of the glutamate receptor antagonist, MK-801, may or may not have reduced osteoclast resorption of the calvarial bone. Though there was not a significant decrease in the amount of osteoclasts per millimeter of bone in the MK-801 infused mice compared to the control mice, there was noticeably less bone resorption and remodeling (Figure 4). The mean number of osteoclasts per millimeter of bone was 1.995 and 3.380 for the MK-801 infused mice and control mice, respectively. Further studies using additional MK-801 doses and/or other receptor antagonists to enhance osteoclast activity and larger sample sizes will help to clarify these results.

Discussion

In this study, an autologous dermal implant was placed on the calvaria of mice to simulate a cholesteatoma. Cholesteatomas lead to a chronic inflammatory process, which commonly leads to localized bone resorption (Chole et al., 2000). Bone resorption on the other hand, occurs as a result osteoclastic activity. The activation and adherence of osteoclasts is influenced by both nitric oxide and glutamate receptors. Results of this study indicate that the glutamate receptor antagonist, MK-801, may be effective in reducing the number of osteoclasts as compared to the control group. Although the difference between the two groups was not significant, a trend in the expected direction was noted. Also, objective observations suggest that fewer osteoclasts were formed on the surface of calvarial bone of MK-801 treated animals. Thus

the MK-801 may not be able to access interior regions of bone, like marrow spaces, where osteoclast recruitment and activation commonly occurs.

Acknowledgements

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Figure Legends

Figure 1 Low resolution photomicrograph of epon sections through mouse calvaria. Dermal implant (DI) is fixed to the calvarial bone (B), centered over the sagittal suture of the parietal plates.

Figure 2 High power photomicrographs of osteoclasts from mouse calvaria. In order to be counted as an active the cell must be adjacent to bone (B) and meet three of the four following criteria: a ruffled border (rb), granular cytoplasm, multiple nuclei (*), and the absence of lamina limitans (arrows).

Figure 3 Histogram showing the means and standard deviations control mice (green) and MK-801 infused mice (red). The mean number of osteoclasts per millimeter of bone was 3.380 and 1.995 for the control mice and MK-801 infused mice, respectively. The means were not significant ($P=0.090$).

Figure 4 Low resolution photomicrograph of sections through the region of the sagittal suture reveal a greater degree of bone erosion (bone = b) in control tissues compared to tissues infused with the glutamate receptor antagonist, MK-801.

Figure 1

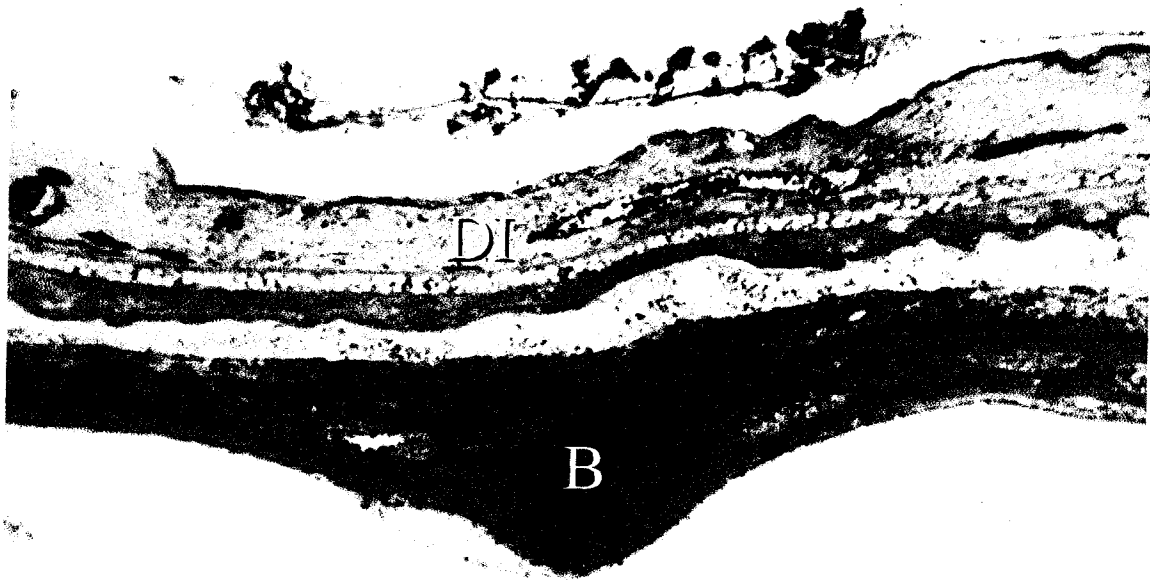


Figure 2



Figure 3

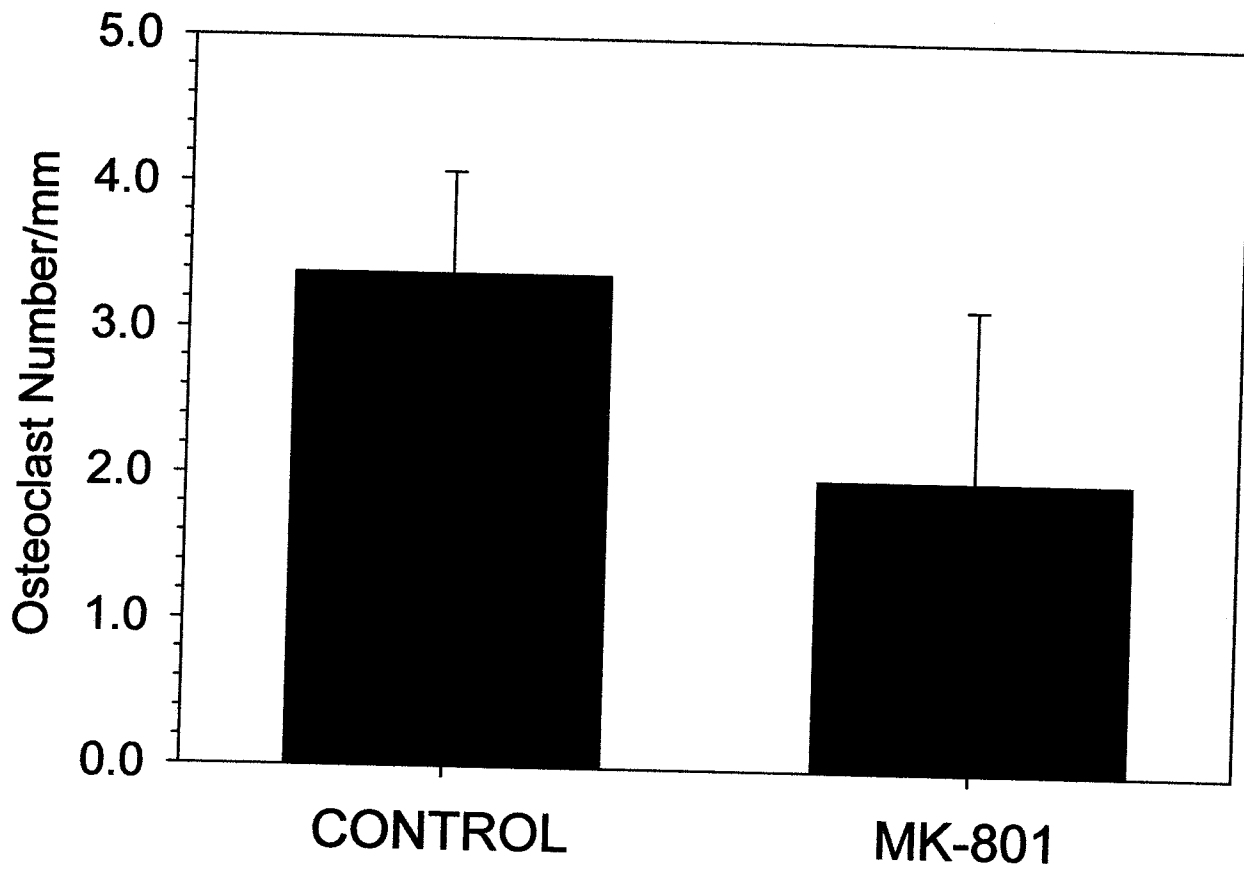
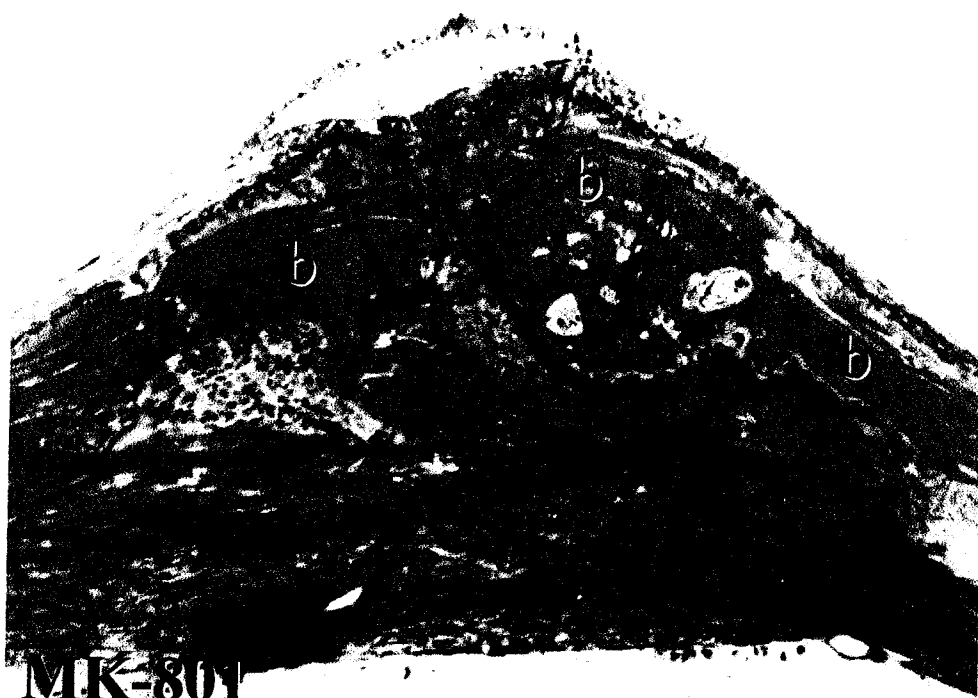


Figure 4



Control



MK-801

Figure 1

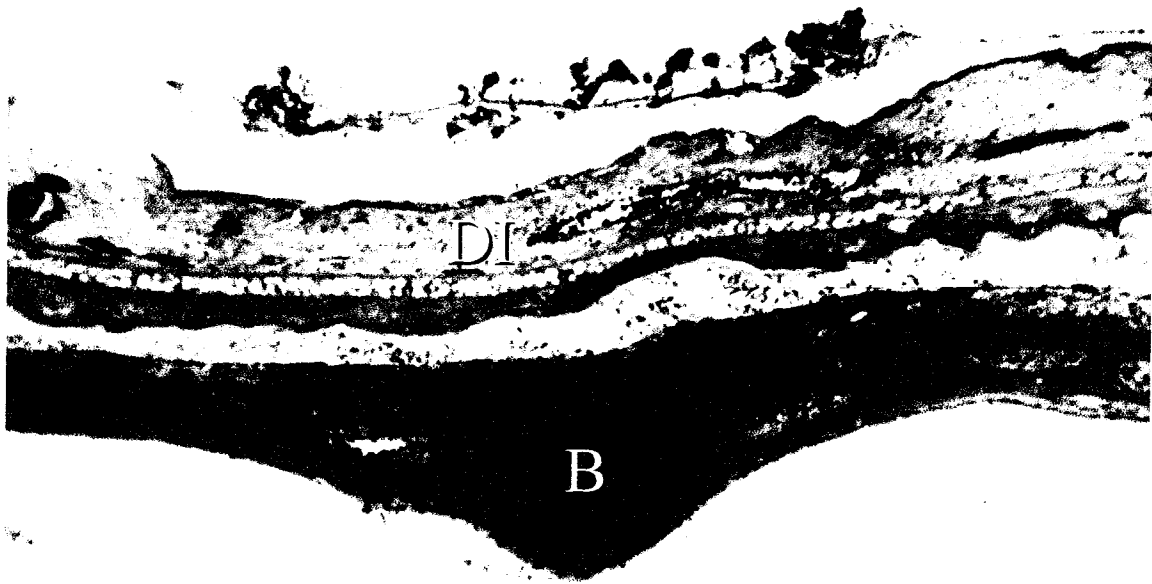


Figure 2



Figure 3

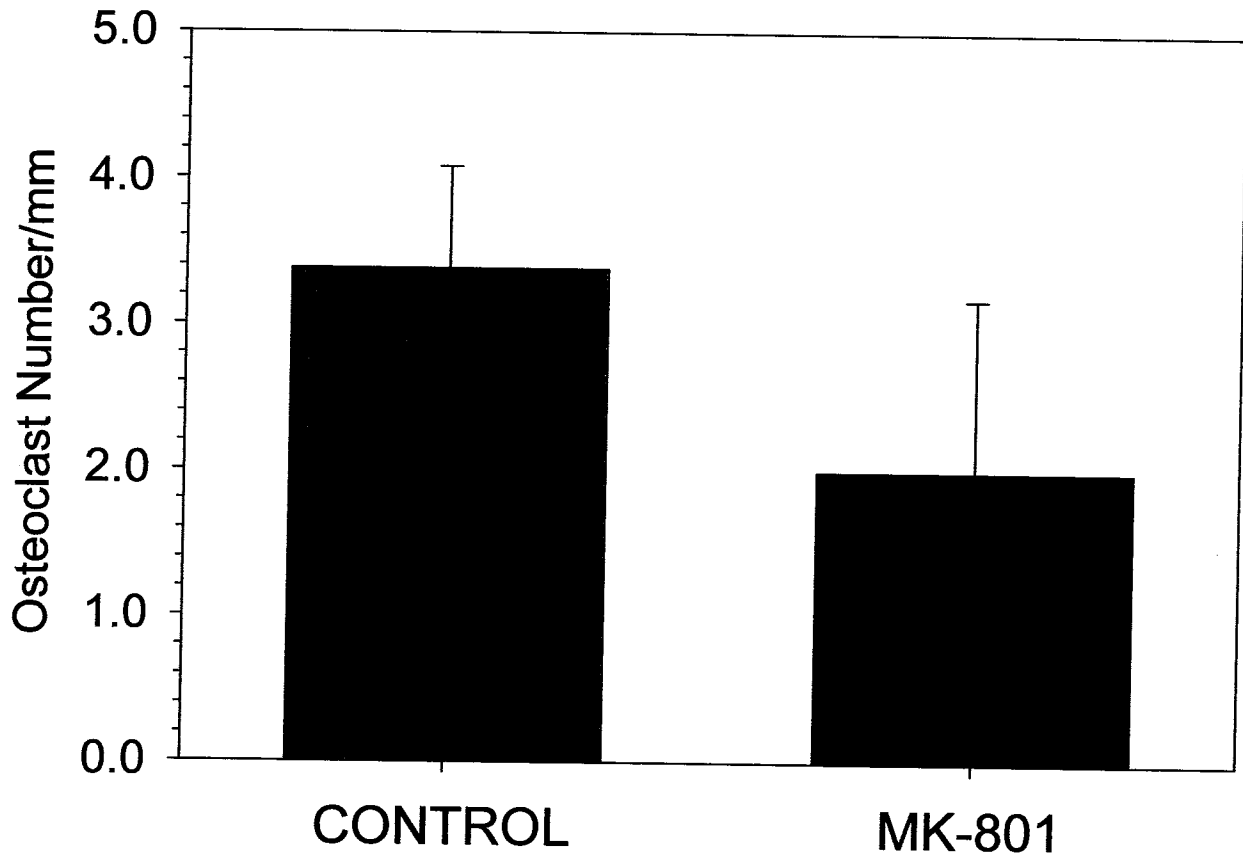
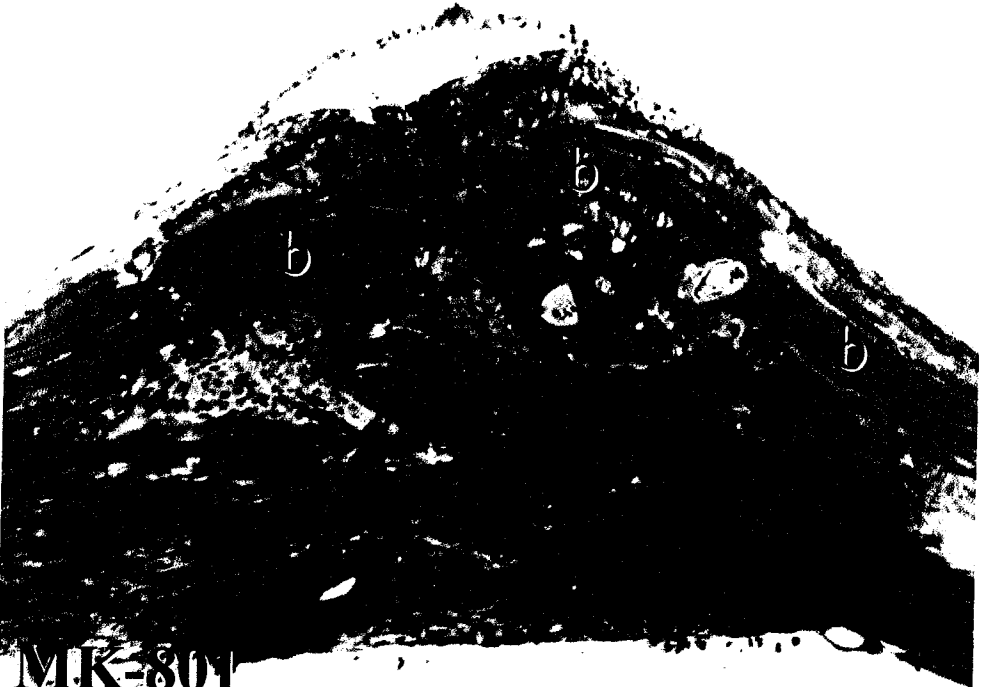


Figure 4



Control



MK-801