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Histopathology in Rat Temporal Bones Resulting from Excess Dietary Fluoride in Conjunction with Suspected Water Born Toxins

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HISTOPATHOLOGY IN RAT TEMPORAL BONES RESULTING FROM EXCESS DIETARY FLUORIDE IN CONJUNCTION WITH SUSPECTED WATER BORN TOXINS

by

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HISTOPATHOLOGY IN RAT TEMPORAL BONES RESULTING FROM EXCESS DIETARY FLUORIDE IN CONJUNCTION WITH SUSPECTED WATER BORN TOXINS

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The occurrence of perinatal deaths, bony extoses, and deafness in dog kennels in certain areas of Michigan led The Upjohn Company and Western Michigan University to suspect fluoride toxicity and/or water born toxins. The present project was designed to examine the ability of excess fluoride ingestion and/or the ability of suspected water born toxins to induce histopathology in Sprague-Dawley rats. An otopathological assessment of the rat temporal bones showed no otopathology nor any changes in cochlear dimensions, however, a quantitative assessment of osteoclastic density showed a large increase in the numbers of osteoclasts of spongy bone in the high fluoride animals.

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Donna Lee Healy

HEALY, DONNA LEE

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INTRODUCTION

People have intermittently been aware of the hazards of fluoride for many centuries. Fluoride toxicity was noted in sheep in Iceland almost 1000 years ago and was attributed to volcanic eruptions (Shupe, 1972). Fluoride compounds are widely distributed in the environment, however fluoride rarely occurs in the free state. It is the 17th most common element and constitutes 0.032% of the earth's crust (Shupe, 1972).

Fluoride was first discovered by Morichini in 1805 in the tusks of fossilized elephants, and later Morichini found fluoride in human tooth enamel. In 1807 Berzilius reported fluorine in human teeth and bones (Gabovich et. al, 1977). In the same year Middleton postulated that fluorine enters the body through water (Gabovich et. al., 1977). To test his hypothesis, Middleton analyzed fragments of water pipe deposits, stalagmites, and tea kettle deposits and found traces of fluoride in each.

Awareness of fluoride increased throughout the 1800's and early 1900's due to the fact that a number of scientists suffered tragic deaths when working with or producing fluorine gases. Since 1950, a number of studies have shown that fluoride is a permanent component of both hard and soft tissues of the human body. Table 1 gives data on the fluoride content of various tissues for different age groups (Gabovich, 1950).

The distribution of fluoride in tissues changes in proportion to the amount of fluoride ingested. In addition, a pronounced tendency for an increase in fluoride content of the tissues with increasing age was observed. The age relationship is due to a slower metabolism of

Table 1. Fluoride Content of Various Tissues

	Age Group (Fluoride in ppm*)		
Tissue	5 - 10 years	40 years	70 - 90 years
Brain	0.16	0.67	0.84
Skeletal Muscle	0.14	0.25	0.31
Heart Muscle	0.49	0.72	--
Blood	0.13	0.36	--
Lungs	0.31	0.52	0.71
Liver	0.24	0.38	0.67
Kidney	0.41	0.68	0.54
Spleen	0.28	0.81	--
Thyroid Gland	0.28	0.69	6.35
Pancreas	0.61	0.84	2.77
Adrenals	0.72	1.73	4.10
Skin	--	3.95	--
Epidermis	--	37.00	--
Hair	--	52.80	72.30
Fingernails	67.00	75.40	89.00
Bones	310.00	450.00	882.00
Teeth	105.00	207.00	283.00

*parts per million

older individuals which leads to a build up of fluoride in the tissues.

Gabovich (1977) has also reported a constantly fluctuating value for blood plasma levels of fluoride. The values ranged from 0.07 ppm to 0.2 ppm, and correlated with the time of day that the measurements were taken. Early morning measurements (before a meal) tended to be very low, while measurements after a meal showed sharp increases.

Gabovich did not study placental tissue, but in 1955 Felltman and Kosel showed that fluoride does cross the placenta and that sizeable amounts of fluoride can be transfered to the human fetus. According to studies by Gedalia et. al. in 1964, the placenta plays an active role in accumulating and transfering fluorine to the human fetus.

Gabovich has also confirmed the transfer of fluoride through the breast milk (1977).

Since 1960 fluoride has been shown to have positive affects on caries reduction in individuals of all ages when it is added to the drinking water and has received wider attention than other fluoride studies. Drinking water fluoridation has been widely implemented over the past 30 years on the basis of reported caries reduction.

The positive correlation between drinking water fluoridation and prevention of caries stimulated interest in many new areas of research. For example, many studies have been done that measure the fluoride content of bone, teeth, plasma, and other tissues and the measurements have been compared to the level of fluoride in the diet (Elkstrand, 1977). In addition it became important to know how much fluoride would provide maximal caries reduction. Studies revealed the optimal fluoride concentrations in the drinking water to be 0.3 to 1.2 ppm.

Excess fluoride in the drinking water can have adverse effects on the appearance and development of the teeth. In areas where the fluoride concentration exceeds 1.5 ppm in the drinking water, the teeth take on a gray or brown mottled or spotted appearance. Teeth can also become discolored; gray, yellow, or brown, and severe cases show black spots on the enamel. If the fluoride concentration increases beyond 10 ppm (or if there is long term exposure to fluoride concentrations that are greater than 1.5 ppm) pitting of the enamel and attrition (wearing down of the teeth) occurs. Permanent teeth do not remain firmly attached to the bone, and they tend to

erupt early (underdeveloped and hypocalcified). Concurrently, as fluoride concentration in water increases, the fluoride concentration in tooth enamel and dentin increases proportionally (Elkstrand et. al. 1981). Permanent teeth are far more severly affected than are temporary teeth. The effects on teeth due to excess fluoride is known as dental fluorosis (Shupe, 1963).

In 1980 Smid showed an increase in the enamel width and strength of rat molars for animals that had been given five injections of three milligrams per kilogram sodium fluoride over 24 hours and sacrificed two hours following the final injection.

The present project is concerned more with the skeleton than with the teeth or other tissues. Skeletal fluorosis is the more dangerous and permanent condition resulting from excess fluoride intake. The higher the fluoride concentration in the diet, the faster the symptoms progress, and the more severe the effects.

In 1974 Faccini et. al. examined nine patients who had lived in a naturally high fluoride environment for at least six years (the concentration of fluoride was not given) and the majority of the patients had lived there for 20 or more years. The investigators examined iliac crest biopsy specimens and found significant changes in the bone. There was a substantial increase in bone resorbtion correlated with severe radiological changes. An increase in the fluoride content of the plasma and bone was clearly evident in these patients. The longer the patient had lived in the high fluoride environment, the more advanced were the symptoms of skeletal fluorosis. Histological studies on the nine patients revealed uncharacteristic bone for-

mation and an increase in new bone forming surfaces. Faccini and his investigators also noted a conspicuous number of osteoclasts (cells which resorb bone) and observed that tissues located in close proximity with the bone (tendon and muscle) had undergone calcification.

In 1981 Ream showed larger bone dimensions (lengths and widths) and an increase in the width of the epiphyseal plate in rats fed 150 ppm fluoride in the drinking water for 10 weeks. Ream's study stressed that there was a direct correlation between the width of the epiphyseal plate and the future length and width of the bone.

Ream and Pendergrass showed in 1982 that there was an increase in the thickness of the periosteum of adult rats placed on a high fluoride diet (150 ppm). The rats showed a significantly increased level of woven bone which indicated high rates of bone resorption.

In 1980 Leonard reported a decrease in bone resorption in rats that were orally swabbed five times per week with 10% stannous fluoride. Leonard's findings indicated a harder material being incorporated into the bone (the fluoride ions displace hydroxyl ions of hydroxyapatite in bone leading to changes in crystallinity) and a sharp increase in the bone crystallinity.

An important function of bone is to help maintain mineral homeostasis throughout the body. Bone is normally being resorbed slowly to supply the blood plasma with calcium ions and calcium from the diet is then incorporated into bone to maintain the integrity of the bone tissue. This overall process helps maintain normal calcium ion homeostasis.

The component in bone that accounts for bone density and hardness

is a compound called hydroxyapatite. When high levels of fluoride are ingested through either food or water, the hydroxyl ions of hydroxyapatite are displaced by fluoride. This leads to the formation of fluoroapatite (an extremely stable compound) and continued ingestion of high concentrations of fluoride can lead to a substantial amount of hydroxyapatite being replaced by fluoroapatite. The incorporation of fluoroapatite increases the density of bone and eventually leads to an increase in the overall size.

Since fluoroapatite is such a stable compound, it is much more difficult to continue normal bone metabolism. Initially the difficulty in resorbing fluoroapatite triggers the osteoclasts to locate and resorb any available hydroxyapatite. When hydroxyapatite has been fully replaced by fluoroapatite, resorbtion becomes increasingly difficult and a decrease in the plasma calcium level occurs. Low calcium levels trigger secretion of parathyroid hormone, which stimulates the osteoclasts to increase their activity and/or numbers and this eventually leads to an increase in the plasma calcium levels to normal.

The importance of maintaining plasma calcium ion concentration illustrates that fluoride intake must be carefully controlled to avoid the symptoms seen in dental and skeletal fluorosis.

An example of accidental excessive fluoride intake has been observed in Allegan, Michigan. For the past 12 years, Shetland Sheepdogs in the Moribrook Kennel in Allegan have shown a marked increase in perinatal deaths. Since 1977, the dogs have shown a high incidence of birth defects, particularily involving the bones.

Other related health problems included discolored teeth and bony extoses (tumors). In addition, individuals who have been involved in the care of these animals feel that as many as 75% are partially deaf and/or have vestibular (balance) problems.

Chemical analysis by The Upjohn Company revealed that the dog food being used contained extremely high concentrations of fluoride (460 ppm). Rock phosphate was being incorporated into the dog food as a dietary source of calcium and phosphate. Rock phosphate is a relatively indigestible compound (T. Marks, personal communication, 1983) and a large amount of the substance must be incorporated into the dog food to ensure that the dogs receive the recommended amounts of calcium and phosphate. Also present in rock phosphate is fluoride and when added to dog food, the resultant fluoride concentration is usually 20 to 25 ppm. The rock phosphate used in the high fluoride dog food had a 20 fold increase in fluoride concentration resulting in a 460 ppm fluoride concentration. It was reported that the manufacturer of the dog food was not initially aware of the high fluoride content of the rock phosphate (T. Marks, personal communication, 1982) but subsequently took it off of the market when the results of the fluoride assays were revealed.

In relation to the problems observed at Moribrook, there are other variables that should be taken into consideration. The water source at the kennel was checked and rechecked and no excess fluoride was found, the water was found acceptable for drinking in all tested respects. Contact with the ground could have exposed the dogs to substances that may have contributed to the problems seen at the kennel.

In terms of ruling out fluoride as a causitive agent for the problems seen at the kennel, the high fluoride dog food was removed from the dogs' diet two years ago, yet the same problems of perinatal deaths, bony extoses, discolored teeth, and deafness remain and have in fact increased substantially over the past two years.

In an effort to learn more about the many problems seen at the Moribrook Kennel, Tom Marks and other interested individuals have utilized the kennel for several studies to investigate the possibility of fluoride as the causitive agent. One study utilized 20 dogs randomized into high and normal fluoride groups, this study is still in progress. A second study utilized 108 rats, 72 females and 36 males. The rats were randomized into four treatment groups with 18 females and nine males per group.

The rats assigned to group A received high fluoride (460 ppm) dog food and the kennel well water. Group B was assigned high fluoride dog food and distilled water. Group C rats received normal fluoride dog food (24 ppm) and kennel well water, group D rats received normal fluoride dog food and distilled water. The two water sources for the groups were intended to establish whether or not toxins were present in the water that may not have been detected by chemical analysis by The Upjohn Company.

The primary concern of the present project is with a representative sample of the temporal bones of these rats. Since as many as 75% of the dogs have been reported to be partially deaf and/or to have vestibular problems; and since it is known that the birth defects and health problems that the dogs have exhibited involve the skeletal

system, the temporal bone offers an excellent opportunity for the anatomical assessment of both major problem areas. Temporal bones contain both compact and spongy bone types as well as the otic capsule, the most compact bone in the body, and there are two diarthoid joints in the middle ear.

For the present investigation, three males and three females from each of the assigned groups (totaling 12 males and 12 females) and three offspring from each of the three females were utilized. The rat study was limited to the affects of high fluoride in the food and the affects of the water, ground contact was not allowed.

The next step in the present study was to determine exactly what was reasonable to expect and examine as a result of the two variables. First a thorough otopathological assessment would be done to determine the presence or absence of otopathology in the temporal bones. This would assess any otopathology that could possibly account for losses in hearing or vestibular problems that could be detected with the light microscope.

The effects of fluoride were clarified through a review of the literature, and the information was utilized to determine what could be examined. This investigator decided to examine the following characteristics in terms of the bony tissue:

(1) Measurement of the lengths and widths of the cochleas in two sections closest to being midmodilar. Based upon Ream's results of 1981, it is reasonable to propose greater lenths and/or widths of the cochleas for rats on the high fluoride diet.

(2) Determination of the number of osteoclasts in the spongy part of the temporal bone bridge using a number of microscopic fields. Based upon Faccini's work in 1974 it is reasonable to expect an increase

in the number of osteoclasts in the temporal bones of the rats on the high fluoride diet.

MATERIALS AND METHODS

Thirty-six male and seventy-two female Sprague-Dawley rats were randomly divided into four groups of nine males and 18 females per group. Two groups were placed on high fluoride diets and two groups were placed on normal fluoride diets. A second variable, well water or distilled water, was also incorporated into the design so that the following groups were formed:

Group A - high fluoride food and well water

Group B - high fluoride food and distilled water

Group C - normal fluoride food and well water

Group D - normal fluoride food and distilled water

The high fluoride food contained 460 ppm fluoride and the normal fluoride food contained 24 ppm fluoride (T. Marks, personal communication, 1983). Food and water were provided ad libitum.

All animals were kept on their specific diets until the time of sacrifice. Eighteen and a half weeks after the groups were formed, the males and females were mated within their respective groups; one group A male was mated to two group A females, one group B male was mated to two group B females, etc. The males and females were placed together for 21 days.

Following the mating period, 12 males were removed for this investigator's specific study, sacrificed by CO_2 and their temporal bones removed in block (right and left bones attached to each other). The

remainder of the males were removed by the Upjohn Company.

Three weeks after the birth of the pups, 12 females and three pups from each litter were removed for this investigator's specific study. These animals were sacrificed by cervical dislocation, and the temporal bones were removed in block. The remainder of the rats were removed by the Upjohn Company.

Histological Preparation of the Tissue

Following removal of the 60 pairs of temporal bones, each pair was placed in Heidenhain-Susa fixative solution for 48 hours (Appendix A). The temporal bones were then washed in running tap water for 24 hours, followed by decalcification. The decalcification consisted of placing the temporal bones in a five percent trichloroacetic acid (TCA) solution. The five percent TCA was renewed every two days until four days after complete decalcification. Complete decalcification was determined by a chemical endpoint test (Appendix B). Two to four weeks were required for complete decalcification. Following decalcification the temporal bones were washed in running tap water for 24 hours then neutralized in five percent sodium thiosulfate for 24 hours (Appendix B). The bones were next subjected to serial dehydration (Appendix C) to replace the water in the tissues with ethyl alcohol.

Celloidin was chosen as the embedding material because of its characteristic for minimum shrinkage as it hardens. This characteristic gives good preservation of the relative positions of the anatomical structures of the temporal bone. A serial embedding process

was used to infiltrate the temporal bones with celloiden (Appendix D). Following completion of the embedding series, the jars containing the temporal bones were left partially open for eight to 12 hours per day, and resealed at night to allow for slow evaporation of the alcohol/ether solvent. This slow evaporation process resulted in even hardening of the celloidin. When the correct firmness of the celloidin was obtained (just after the sticky quality disappeared), the temporal bones were removed from the jars and the excess celloidin was trimmed away. Fifteen percent celloidin was then used as glue to attach each temporal bone block to a wood block. The temporal bone block with its attached wood block was then exposed to chloroform for 24 hours to cause accelerated hardening of the celloidin glue, and to give an additional degree of hardening to the temporal bone block. The blocks were then stored in 80% ethyl alcohol until the time of sectioning.

Sectioning

Each temporal bone block was placed in the chuck of an American Optical model 860 sliding microtome with a constant 80% ethyl alcohol drip to keep the celloidin from drying excessively. The block was then trimmed from superior to inferior to the level of the ampullation of the superior semicircular canal, and these sections were all discarded. From the beginning of the ampulla sections were cut at 20 micrometers and counted. Out of every five sections, the first and last were kept for analysis and the others were discarded. Sectioning was completed at the level of the end of the crista of the posterior canal. As the sections were cut, each one was rolled out flat on

the microtome blade with a camel's hair brush, then placed between two peices of onion skin paper, and stored in 80% ethyl alcohol.

Staining

Every fifth temporal bone section was stained with hematoxylin and eosin (Appendix E) and the other set of sections was kept for special staining procedures, and to supplement in any area where additional detail was desired.

Mounting

Stained sections were centered individually on standard size glass slides and excess celloidin was removed with a razor blade. Two to three drops of permount were placed on the slide, and a 22 by 40 millimeter glass coverslip was placed over the section. Excess permount was removed with xylene and the slides were placed under weights for two weeks to insure that the sections remained flat between the glass plates and to minimize the occurance of air bubbles.

Analysis

Otopathological Assessment

Each temporal bone was analyzed using a model 150 American Optical microscope and a double blind assessment paradigm. An example of a complete otopathological assessment for one pair of temporal bones is provided in Appendix F.

In general, the following structures were viewed at 40X and 100X, and selected aspects of the various structures were viewed at 400X:

-superior semicircular canal
-superior crista
-malleus
-incus
-stapes
-middle ear cavity
-lateral semicircular canal
-lateral crista
-utricle
-saccule
-cochlea
-posterior semicircular canal
-posterior crista
-seventh and eighth cranial nerves
-specific bony structures within the temporal bone

Assessment of Bone

Each temporal bone was assessed using a model 150 American Optical microscope, an eyepeice micrometer, and a double blind assessment paradigm. The following aspects were examined:

-Lengths and widths of the cochlea for the two most midmodilar sections were recorded in micrometers for both right and left sides (viewed at 400X).

-The number of osteoclasts were counted in two predetermined fields of one section of the body of the sphenoid bone. The section chosen for osteoclast counting was at the level of the middle of the incudomalleal joint (the same level for each temporal bone was used to maintain consistency). The body of the sphenoid bone contained the predetermined fields and was assessed at 400X.

Results of the assessment of bone for each individual temporal bone are tabulated in Appendices G and H.

Statistics

A t-test was used to assess the level of significance for the correlations between the sexes and between ages. An f-test was used to assess the level of significance for the correlations between groups (the f-test is used for data that has more than one degree of freedom).

RESULTS

Otopathological Assessment

Except for animal number 170 B 2, all temporal bones were found to be free of otopathology. Animal 170 B 2, a male pup, showed a break in the superior semicircular canal. The break showed signs of healing due to infiltration of connective tissue into the area. A severe infection had invaded the perilymphatic space of the nonampullated end of the superior semicircular canal and the infection had migrated to the utricle. Approximately 90% of the temporal bones had a number of hemorrhagic areas, probably resulting from the removal process, and therefore not significant in terms of otopathology.

Assessment of Bone

Lengths and Widths of the Cochlea

In Table 2a, the mean lengths and widths of the cochleas are shown (in micrometers) according to group (A, B, C, D). The p values show no significant differences between the four groups.

Table 2a. Mean Cochlear Dimensions (micrometers)

GROUP	LENGTH	WIDTH
A	1997.4	1830.1
B	1956.1	1797.3
C	1978.7	1816.6
D	1994.1	1821.0

p values range from 0.0642 - 0.7838

A complete table of all cochlear lengths and widths for each temporal bone can be found in Appendix G.

When the lengths and widths were compared between the two age groups (Table 2b) a statistically significant difference was found in the widths only. This was not expected because the cochlea should be full size (or close to full size) at birth (Bélanger, 1956).

Table 2b. Mean Cochlear Dimensions (micrometers)

GROUP	LENGTH	WIDTH
Adult	1985.1	1711.1
Pup	1980.8	1866.2

p value equal 0.0001 when comparing widths

When the lengths and widths were compared according to sex, no statistical significance was found. The results are tabulated in Table 2c.

Table 2c. Mean Cochlear Dimensions (micrometers)

SEX	LENGTH	WIDTH
F	1976.8	1807.5
M	1987.1	1824.7

p value equal 0.0949

Number of Osteoclasts

Table 3a shows the mean number of osteoclasts per microscopic field as calculated for each group. The p value was calculated comparing groups A and B to groups C and D. Group A did not differ statistically from group B and group C did not differ statistically from

group D. The results showed a highly significant difference between the two food groups.

Table 3a. Osteoclasts/Field

GROUP	MEAN # OSTEOCLASTS
A	6.8
B	6.3
C	2.6
D	3.5

p value equal 0.0001

A complete table of the number of osteoclasts in each fields for each temporal bone is included in Appendix H.

Table 3b shows the comparisons between the mean number of osteoclasts according to age only. The p value showed that the number of osteoclasts in the adult was significantly higher than the number of osteoclasts in the pup. This was the expected result since adults rely on osteoclastic activity to maintain calcium ion homeostasis, whereas pups receive much of their calcium supply from the breast milk (Wilson, personal communication, 1983).

Table 3b. Osteoclasts/Field

GROUP	MEAN # OSTEOCLASTS
Adult	5.8
Pup	4.2

p value equal 0.005

Table 3c illustrates the mean number of osteoclasts according only to sex. The results were not statistically significant.

Table 3c. Osteoclasts/Field

SEX	MEAN # OSTEOCLASTS
F	5.2
M	4.5

p value equal 0.0405

Figure 1 below depicts a representative field containing osteoclasts seen in rats receiving high fluoride food (460 ppm).

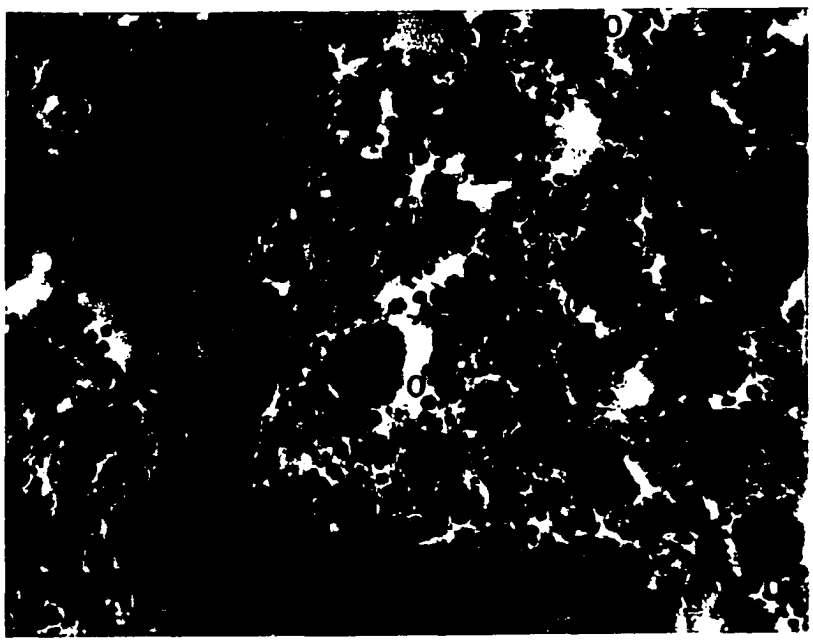

Figure 1. Representative field containing osteoclasts from a rat receiving high fluoride. Note five osteoclasts (O) in the field. 400X.

Figure 2 below depicts a representative field containing one osteoclast in rats receiving normal fluoride food (24 ppm).

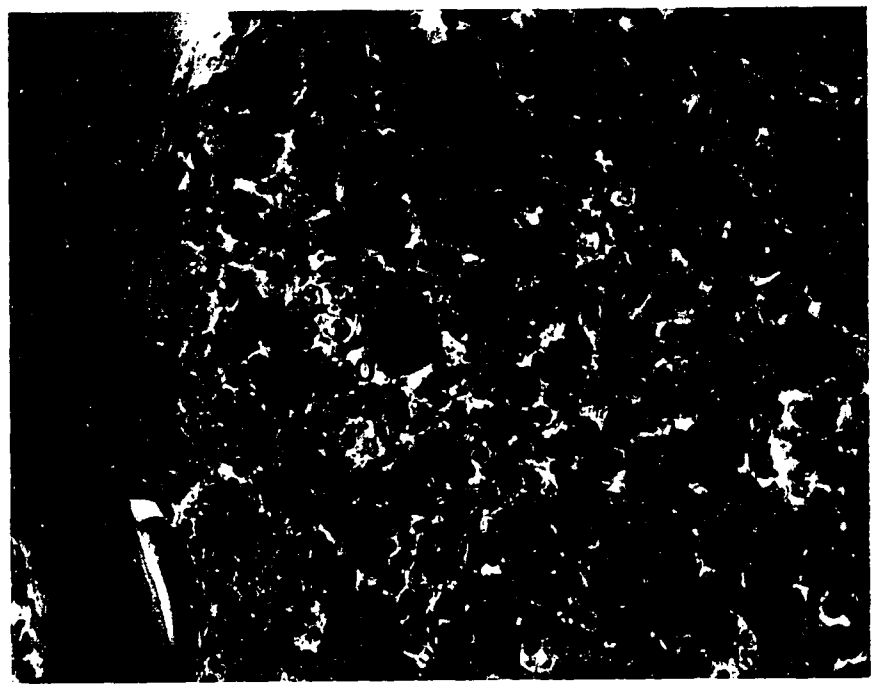

Figure 2. Representative field containing one osteoclast from a rat receiving normal fluoride. Note the single osteoclast (O) in the field. 400X.

DISCUSSION

Otopathological Assessment

The otopathological evaluation consisted of assessing the auditory and vestibular structures within the temporal bone. Appendix F depicts an otopathological assessment of a randomly chosen temporal bone.

All animals were found to be free of otopathology except for animal number 170 B 2 male which had a severe infection of the perilymph of the nonampullated end of the superior semicircular canal.

The high frequency of hemorrhage present in the majority of the temporal bones was most probably due to blood vessel disruption at the time that the bones were removed from the skull. This result is therefore not significant in terms of otopathology.

The one animal that showed some pathology was a male pup whose mother received high fluoride. The pathology seen in this pup (170 B 2 male) included a break across the superior end of the osseous portion of the nonampullated end of the superior canal. The break showed signs of healing as indicated by infiltration of connective tissue in the area of the break. A severe infection had developed in the perilymphatic space of the canal and had migrated into the utricle. The statistical evidence does not support the concept that fluoride had any affect on this particular rat's condition, otherwise more than one of the 30 animals receiving high fluoride would probably have shown similar or related effects.

Assessment of Bone

Lengths and Widths of the Cochlea

After reviewing related literature, this investigator hoped to find

an increase in the cochlear lengths and widths in the high fluoride groups as compared to the normal fluoride groups. After twice measuring these dimensions, the high fluoride groups showed no significant difference when compared to the normal fluoride groups (p values greater than 0.06). The only significant result which was obtained resulted from comparing the adults as an entire group to the pups as an entire group. The pups were found to have significantly wider (not longer) cochleas than the adults. Since there was no difference in the size of the cochleas when comparing the two dietary groups, and since the measurements were double checked, the difference exists for reasons that are unclear at the present time. No significant difference was found in the mean lengths and widths of the cochleas between the sexes, a fact which is in agreement with expectations since males and females received the same treatments, and the general structure of bone does not differ between the sexes (Collins, 1966).

Number of Osteoclasts

The number of osteoclasts from the two fields of sphenoid bone for the high fluoride groups were found to be significantly higher than the number of osteoclasts found in the two fields of sphenoid bone of the normal fluoride groups. The mean number of osteoclasts for the high fluoride group was 6.8 per field, as compared to the normal fluoride group which had 3.2 osteoclasts per field. This difference in osteoclast numbers in consistent with Faccini's observations in 1974 in which he commented on the presence of "conspicuous numbers of osteoclasts." Fig. 1 depicts a representative field containing osteoclasts in rats receiving high fluoride. Fig. 2 depicts a representative field

containing one osteoclast in rats receiving normal fluoride food.

The reported physiological effects of high fluoride intake which has been reported in the literature support the possible occurance of increased numbers of osteoclasts (Faccini, 1974; Elkstrand, 1981; Leonard, 1980; Ream, 1981). Since fluoroapatite in bone (from high fluoride intake) is more difficult to catabolize than normal hydroxyapatite, there is a decrease in the plasma calcium ion level. This stimulates an increase in the secretion of parathyroid hormone which increases the number of osteoclasts, so that more bone can be catabolized. Thus the plasma calcium ion concentration is maintained at normal levels but with elevated numbers of osteoclasts.

Comparing the ages only, the adults were found to have a statistically higher number of osteoclasts than the pups. This is a reasonable expectation in light of the fact that the pups have less need for bone catabolism to supply their plasma calcium ions. The pups are still forming new bone from cartillage and they receive much of their calcium from the breast milk (Wilson, personal communication, 1983).

The number of osteoclasts were compared between the sexes and the resultant differences were not found to be statistically significant. This was expected since there were no differences between the general bone structure of males and females in the literature.

CONCLUSION

In summary, the results obtained in the present project show that excess fluoride caused a statistically significant increase in the number of osteoclasts found in spongy bone of treated rats. Statistical analysis did not reveal a significant difference between the groups receiving kennel well water and groups receiving distilled water, which showed no detectable water born toxins, and is also in agreement with the chemical analysis by the Upjohn Company.

This investigator beleives that the histopathological effects of fluoride should be studied in closer conjunction with the physiological effects. For example, if a similar project was to be undertaken this investigator would remove not only the temporal bones, but also the femurs and the parathyroid glands for study. The glands would be utilized to assess the effects of fluoride on the gland activity, and the femur would be assessed to establish the effects of fluoride on bone thickness as illustrated in Ream's studies. In addition, plasma parathyroid hormone levels would be assessed before ingestion of excess fluoride and a number of times during the treatment, as well as plasma and bone fluoride levels.

Although it is clear from related literature that trace amounts of fluoride are a beneficial component for the human body, careful control of fluoride levels is necessary to avoid the physiological and histological effects of excess fluoride ingestion.

APPENDIX A

Fixative Solution

Heidenhain-Susa Fixative

Stock Solution:	Mercuric Chloride	45 grams
	Sodium Chloride	5 grams
	Distilled Water	800 milliliters
	Trichloroacetic Acid	20 grams
	Glacial Acetic Acid	40 milliliters
Working Solution:	Stock Solution	240 milliliters
	37% Formaldehyde	60 milliliters

APPENDIX B

Decalcification and Neutralization Solutions

5% Trichloroacetic Acid

Trichloroacetic Acid ---------- 5 grams
Distilled Water --------- 100 milliliters

Chemical Endpoint Test

5% Ammonium Oxalate: Ammonium Oxalate ---------- 5 grams
Distilled Water ---- 100 milliliters

5% Ammonium Hydroxide: Ammonium Hydroxide --------- 5 grams
Distilled Water ---- 100 milliliters

Mix 2 milliliters of the solution that the temporal bone is decalcifying in with 1 milliliter of 5% ammonium oxalate, and 1 milliliter of ammonium hydroxide. The formation of a white precipitate indicates that the decalcification is incomplete. Allow the mixture to stand for 30 minutes before assessing for a cloudy formation.

APPENDIX C

Serial Dehydration Procedure

Alcohol Dilutions

To obtain the desired concentration of alcohol the following formula was used:

$$\frac{\text{\% alcohol desired}}{\text{\% alcohol available (95\%)}} = \frac{\text{amount of available needed (95\%)}}{\text{total volume of alcohol desired}}$$

Ether-100% Ethyl Alcohol

Ether (Anhydrous Ethyl) ---------- 10 milliliters
100% Ethyl Alcohol ---------- 10 milliliters

Serial Dehydration Process

1) 50% Ethyl Alcohol ---------- 24 hours
2) 70% Ethyl Alcohol with 1 ml Saturated Iodine* ---------- 24 hours
3) 80% Ethyl Alcohol with 1 ml Saturated Iodine* ---------- 24 hours
4) 95% Ethyl Alcohol ---------- 24 hours
5) 95% Ethyl Alcohol ---------- 24 hours
6) 100% Ethyl Alcohol ---------- 24 hours
7) 100% Ethyl Alcohol ---------- 24 hours
8) Ether-100% Ethyl Alcohol ---------- 24 hours
9) Ether-100% Ethyl Alcohol ---------- 24 hours

*The saturated iodine is for the purpose of removing excess mercury chloride which accumulates in the tissues during fixation. This eliminates unnecessary histological artifact caused by mercury deposits in the tissue, and also serves to minimize damage to the microtome knife which would be caused by excess mercury at the time of sectioning.

APPENDIX D

Serial Embedding In Celloidin

Celloidin Solutions

1.5% Celloidin:	Celloidin	1.5 grams
	Ether-100% Ethyl Alcohol	100 milliliters
3.0% Celloidin:	Celloidin	3.0 grams
	Ether-100% Ethyl Alcohol	100 milliliters
6.0% Celloidin:	Celloidin	6.0 grams
	Ether-100% Ethyl Alcohol	100 milliliters
12.0% Celloidin:	Celloidin	12.0 grams
	Ether-100% Ethyl Alcohol	100 milliliters

Serial Embedding Process

1)	1.5% Celloidin	1 week
2)	3.0% Celloidin	2 weeks
3)	6.0% Celloidin	2 weeks
4)	12.0% Celloidin	3 weeks

APPENDIX E

Harris's Hematoxylin and Eosin Y Staining Series

Lugol's Iodine

Potassium Iodide	4 grams
Iodine Crystals	2 grams
Distilled Water	100 milliliters

Harris's Alum Hematoxylin

Hematoxylin	5 grams
Absolute Ethyl Alcohol	50 milliliters
Aluminum Ammonium Sulfate	100 grams
Distilled Water	1000 milliliters
Mercuric Oxide	2.5 grams

Acid Alcohol

Concentrated HCl	2 drops
Absolute Ethyl Alcohol	100 milliliters

Ammonia Water

Concentrated Ammonium Hydroxide	10 drops
Distilled Water	100 milliliters

1% Alcoholic Eosin

Eosin Y	10 grams
Distilled Water	50 milliliters
95% Ethyl Alcohol	940 milliliters

Absolute Ethyl Alcohol and Chloroform

Absolute Ethyl Alcohol	30 milliliters
Chloroform	30 milliliters

Xylene and Terpinol

Xylene	10 milliliters
Terpinol	10 milliliters

Hematoxylin and Eosin Staining Procedure

1) Lugol's Iodine Solution ---------- 10 minutes
2) 5% Sodium Thiosulfate ---------- 5 minutes
3) Tap Water ---------- 2 changes, rinse
4) Harris's Hematoxylin ---------- 12 minutes
5) Tap Water ---------- 2 changes, rinse
6) Acid Alcohol ---------- 2 dips
7) Tap Water ---------- rinse
8) Ammonia Water ---------- 3 changes, 1 minute each
9) Tap Water ---------- 2 changes, rinse
10) Eosin Y ---------- 30 seconds to 1 minute
11) 80% Ethyl Alcohol ---------- 3 changes, 1 minute each
12) 95% Ethyl Alcohol ---------- 2 changes, 1 minute each
13) 100% Ethyl Alcohol ---------- 1 minute
14) Absolute Ethyl Alcohol and Chloroform ---------- 1 minute
15) Xylene and Terpineol ---------- 1 minute

APPENDIX F

Otopathological Assessment

Otopathological Assessment: A sample otopathological assessment of double blind study animal number 7 is decoded below. In general, the following structures were assessed at 40 and 100 power:

Deep part of external auditory canal
Tympanic membrane
Middle ear including - middle ear cavity, lining, ossicles, ligaments, muscles, blood supply, bony encasement, nerves, and articulations
Inner ear including - all sensory epithelium, support cells, pillar cells, blood supply, ligaments, membranous labyrinth including intraorgan membranes, osseous labyrinth, nerves, and fluid cavities including pressure assessments

Sample Otopathological Assessment

Double Blind Assessment Number: 7
Decoded Animal Number: 234C3 male

RIGHT SIDE - Slide 5 shows the middle ear cavity to be 80% filled with red blood cells. Slide 10 shows the continuing presence of red blood cells in the middle ear cavity. The malleus appears normal. The scala vestibuli of the basilar turn is approximately 85% filled with a protein coagulate material. Slide 15 shows a normal superior crista with edematous hair cells (this is within the limits of normalcy as can be accounted through fixation). The perilymphatic space of the ampullated end of the superior semicircular canal is 80% filled with protein coagulate. The hemorrhage in the middle ear remains at 80%. The scala vestibuli shows a drop in the amount of protein coagulate to 40%. Slide 25 shows a normal incudomalleal joint. The malleus has an area where it has been broken off. This is probably due to histological artifact due to the cleanness of the break. Slide 30 shows a normal utricle with edematous hair cells (normal accounting for fixation). The otoconia of the utricle appear normal. The middle ear cavity remains 80% filled with red blood cells. The perilymphatic space of the ampullated end of the semicircular canal remains 80% filled with protein coagulate. The neural tissue at this level is normal. Slide 35 is midmodilar. All scala vestibuli show some protein coagulate deposits which vary from 10% to 70%. All of the scala tympani show some protein coagulate deposits which vary from 5% to 20%. The scala media are clear. Assessment of the organ of corti at this level

shows normal appearance of the spiral ganglion, spiral limbus, tectoral membrane, stria vascularis, vestibular membrane, and basilar membrane. The hair cells and support cells appear normal as does the tunnel of corti and associated pillar cells. The perilymphatic space of the saccule is 100% filled with a protein coagulate. The middle ear cavity remains 70% filled with red blood cells. Slide 40 shows a normal lateral crista with edematous hair cells (normal accounting for fixation). Slide 45 shows all of the scala vestibuli and scala tympani continuing with the same amounts of protein coagulate as in slide 35. At this level, the saccule is normal with edematous hair cells (normal accounting for fixation), and normal otoconia. The endolymph of the saccule is 10% filled with a protein coagulate deposit. The perilymph of the saccule contains an 80% protein coagulate deposit. The incudostapedial joint is normal. The middle ear cavity hemorrhage remains. Slide 55 shows a continuing protein coagulate deposit in the scala vestibuli and the scala tympani. The nonampulated end of the superior semicircular canal is broken on the medial side. The break is histological artifact due to the cleanness of the break. Slide 60 shows a normal stapes including crura and annular ligament. The footplate appears normal. Slide 65 shows a 30% protein coagulate deposit in the endolymph of the posterior canal. Slide 90 shows a normal posterior cristae with slightly edematous hair cells (normal accounting for fixation). The round window niche is 40% filled with red blood cells. The cochlear side of the round window membrane is 40% filled with red blood cell deposit, with an additional 5% deposit of protein coagulate. The round window membrane is normal.

LEFT SIDE - Slide 5 shows a normal malleus. The middle ear is clear. The superior crista is normal with normal hair and support cells. Slide 10 shows a normal incudomalleal joint. However, the joint shows some separation on the most medial side. This is due to histological artifact. Other small tears in the tissue are evident in the area which is also histological artifact. The perilymph of the ampulated end of the superior semicircular canal is 10% filled with a protein coagulate deposit. The nonampulated end of the superior semicircular canal is broken at this level. This is histological artifact due to the cleanness of the break. The scala tympani of the basilar turn is 5% filled with a protein coagulate deposit. The scala media of the basilar turn is 100% filled with protein coagulate, and the scala vestibuli of the basilar turn is 65% filled with a protein coagulate deposit. The apical turn is not assessible at this level. Slide 25 shows a normal utricle with normal hair cells, support cells, and otoconia. The perilymph of the saccule is 95% filled with a protein coagulate deposit. The protein coagulate seen in the scala media of the basilar turn has been reduced from 100% to 2%. The lateral crista is normal with edematous hair cells (normal accounting for fixation). Slide 30 shows the endolymph of the saccule to be 10% filled with a protein coagulate deposit. 10% of the volume of the middle ear cavity is filled with red blood cells. Slide 35 shows the saccule to now be 40% filled with a protein coagulate deposit. The middle ear cavity is 25% filled with red blood cells. Slide 40 shows a small

fray (not a break) in the nonampulated end of the superior canal. The middle ear cavity is 50% filled with red blood cells. Slide 45 shows the endolymph of the saccule to be clear of protein coagulate, as is the scala media. The annular ligament of the stapes is normal. The middle ear cavity shows a 60% deposit of red blood cells. This level is midmodilar.. All scala vestibuli and scala tympani are approximately 40% to 60% filled with a protein coagulate deposit. All scala media are clear. Assessment of the organ of corti at this level shows a normal spiral ganglion, spiral limbus, tectoral membrane, stria vascularis, vestibular membrane, and basilar membrane. The hair cells and support cells are normal. The tunnel of corti and associated pillar cells are normal. Slide 50 shows the middle ear cavity to be 70% filled with a sparse deposit of red blood cells (the deposit is not packed). The perilymph of the utricle and saccule are both filled with protein coagulate covering 95% of the perilymphatic space. The crura of the stapes, and the incudostapedial joint appears normal. Slide 55 shows a decrease in the middle ear hemorrhage to 10%, and a 60% protein coagulate deposit remains. Slide 85 shows a normal round window niche with a small deposit of red blood cells covering approximately 5% of the area. The cochlear side of the round window membrane contains a 50% protein coagulate deposit. Slide 90 shows the round window membrane to be normal. The posterior crista is normal with slightly edematous hair cells (normal accounting for fixation).

APPENDIX G

Bony Assessment

Lengths and Widths of the Cochlea

The lengths and widths of the cochlea were measured in the two most mid-modilar sections. Two sections were chosen to increase the accuracy of the measurements. All measurements are in micrometers.

GROUP A

Animal Number and Sex	Left Side		Right Side	
	Length	Width	Length	Width
208CA male	1856.0	1948.8	1740.0	1972.0
	1716.8	1995.2	1740.0	2030.0
218CA male	1716.8	2030.0	1682.0	2006.8
	1682.0	2088.0	1798.0	2053.2
210CA male	1763.2	1809.6	1856.0	2006.8
	1798.0	2088.0	--	--
218 A female	1762.2	1972.0	--	--
	--	--	--	--
218 A female	1914.0	2030.0	1972.0	2006.8
pup	1914.0	1995.2	1948.8	1995.2
218 A male	1798.0	1972.0	--	--
pup	1856.0	1914.0	1786.4	1995.2
218 A female	1856.0	2030.0	--	--
pup	1856.0	2006.8	1682.0	1972.0
204 A female	--	--	1624.0	2030.0
	1740.0	1972.0	--	--
204 A male	1856.0	2030.0	1914.0	2030.0
pup	1972.0	2064.8	1856.0	1995.2
204 A male	1798.0	2030.0	1995.2	2041.6
pup	1832.8	2030.0	2030.0	1995.2
204 A female	1832.8	2030.0	1856.0	1995.2
pup	1879.2	2030.0	1914.0	2030.0
164 A female	1566.0	1983.6	1682.0	1972.0
	1705.2	1948.8	1624.0	1972.0
164 A female	1856.0	1925.6	1972.0	1972.0
pup	1972.0	1914.0	1879.2	1948.8
164 A male	1972.0	1995.2	1995.2	1972.0
pup	1856.0	1995.2	1972.0	2053.2
164 A male	--	--	--	--
pup	--	--	--	--

GROUP B

Animal Number and Sex	Left Side		Right Side	
	Length	Width	Length	Width
A22 B male	1798.0	1995.2	1879.2	2030.0
	1740.0	1983.6	1856.0	2030.0
207CB male	--	--	1682.0	1856.0
	1624.0	1856.0	--	--
44 B male	1763.2	2030.0	--	--
	--	--	--	--
176 B female	--	--	1740.0	1914.0
	1624.0	1995.2	--	--
176 B male	2204.0	1879.2	1624.0	1856.0
pup	--	--	1740.0	1856.0
176 B female	1798.0	1937.2	1763.2	1972.0
pup	1798.0	1890.8	1740.0	1937.2
176 B male	--	--	1774.8	1983.6
pup	1856.0	1960.4	1879.2	2030.0
219 B female	--	--	--	--
pup	1682.0	1983.6	1624.0	1914.0
219 B female	1972.0	1995.2	1856.0	1948.8
pup	1832.8	1995.2	1914.0	2030.0
219 B male	1798.0	1983.6	1856.0	1902.4
pup	1972.0	1960.4	2030.0	1879.2
219 B female	1624.0	1995.2	1624.0	1948.8
pup	1682.0	1879.2	1740.0	1960.4
170 B female	--	--	--	--
	1624.0	2030.0	--	--
170 B male	1798.0	1914.0	1856.0	1937.2
pup	1740.0	1983.6	1879.2	1995.2
170 B male	1948.8	1995.2	1856.0	1995.2
pup	2030.0	2006.8	1914.0	1983.6
170 B female	2088.0	1914.0	1740.0	1972.0
pup	1972.0	1972.0	1740.0	1914.0

GROUP C

Animal Number and Sex	Left Side		Right Side	
	Length	Width	Length	Width
48 C male	1647.2	1925.6	1716.8	1890.8
	1740.0	1972.0	1682.0	1948.8
47 C male	1600.8	1972.0	--	--
	--	--	--	--
249 C male	1682.0	2030.0	--	--
	--	--	--	--
224 C female	--	--	--	--
	--	--	--	--
224 C female	1856.0	1972.0	1856.0	2030.0
pup	1821.2	2030.0	1856.0	2053.2
224 C female	1798.0	1937.2	1832.8	1914.0
pup	1856.0	1972.0	1798.0	1972.0
224 C male	1972.0	1006.8	1914.0	1972.0
pup	1972.0	1995.2	1914.0	1983.6
225 C female	--	--	--	--
	--	--	--	--
225 C male	1798.0	1972.0	1624.0	2064.8
pup	1798.0	1972.0	1740.0	2088.8
225 C male	1972.0	1890.8	--	--
pup	2030.0	1879.2	--	--
225 C female	1798.0	1948.8	--	1995.2
pup	--	--	--	2041.6
234 C female	1856.0	1948.8	1798.0	1995.2
	1624.0	1995.2	1624.0	1832.8
234 C female	1798.0	1972.0	1856.0	1972.0
pup	1832.8	1995.2	1856.0	2006.8
234 C female	1832.8	1995.2	1809.6	1972.0
pup	1856.0	1914.0	1751.6	1972.0
234 C male	1856.0	2018.4	--	2041.6
pup	2088.0	2030.0	1948.8	1995.2

GROUP D

Animal Number and Sex	Left Side		Right Side	
	Length	Width	Length	Width
A23 D male	--	--	--	2030.0
	1740.0	2030.0	1798.0	1995.2
223CD male	1740.0	1972.0	1624.0	1983.6
	1600.8	1995.2	1682.0	2053.2
62 D male	1856.0	1914.0	1740.0	1995.2
	1682.0	1972.0	1624.0	1972.0
234 D female	1658.8	1972.0	1740.0	1995.2
	1740.0	1937.2	1798.0	1972.0
234 D male	1972.0	1972.0	1914.0	2030.0
pup	2030.0	2030.0	2030.0	2030.0
234 D female	1914.0	2030.0	2053.2	1508.0
pup	2030.0	2064.8	2088.0	1450.0
234 D female	1914.0	2030.0	1972.0	2030.0
pup	1960.4	1890.8	1937.2	1972.0
249 D female	1740.0	1995.2	1832.8	1995.2
	--	--	--	--
249 D male	1798.0	1995.2	1856.0	1972.0
pup	1740.0	2064.8	--	1995.2
249 D female	1856.0	2262.0	2030.0	2030.0
pup	1879.2	2227.5	--	--
249 D male	1705.2	1972.0	1879.2	1948.8
pup	--	--	--	--
238 D female	1450.0	2146.0	1647.2	2088.0
	--	--	1600.8	2088.0
238 D male	1682.0	2064.8	--	--
pup	1856.0	2018.4	2064.8	1995.2
238 D male	1682.0	2064.8	1995.2	2018.4
pup	1856.0	2018.4	1995.2	1948.8
238 D female	1832.8	1972.0	1740.0	1972.0
pup	1693.6	2041.6	1798.0	1972.0

APPENDIX H

Bony Assessment

Number of Osteoclasts

The number of osteoclasts were counted from one slide. The anatomical location where the counting was done was the same in all cases to maintain consistancy. The section chosen was at the level of the middle of the incudomalleal joint. Two fields were located at the two posterior corners of the sphenoid bone, as illustrated in Figure 3 below. In some cases the sphenoid bone was not intact. When this occured, the closest location of cancellous bone was used for the two fields.

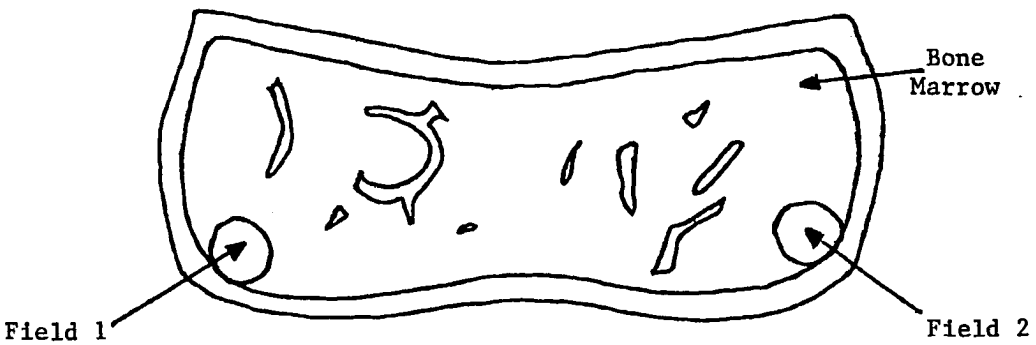

Figure 3: Diagramatic representation of the body of the sphenoid bone. Illustrated are the two posterior corners where osteoclast counting was located.

Number of Osteoclasts

Animal Number	Field 1	Field 2	Animal Number	Field 1	Field 2
GROUP A			GROUP C		
208CA male	4	11	48 C male	6	1
218CA male	5	6	47 C male	3	2
210CA male	7	5	249 C male	-	-
218 A female	8	7	224 C female	2	3
218 A female*	5	5	224 C female*	4	1
218 A female*	8	7	224 C female*	1	1
218 A male*	5	4	224 C male*	4	0
204 A female	10	9	225 C female	2	3
204 A male*	5	4	225 C male*	2	2
204 A male*	6	8	225 C male*	1	4
204 A female*	7	11	225 C female*	3	3
164 A female	8	6	234 C female	2	4
164 A female*	5	8	234 C female*	1	2
164 A male*	9	8	234 C female*	4	5
164 A male*	-	-	234 C male*	5	2
GROUP B			GROUP D		
A22 B male	5	5	A23 D male	2	1
207CB male	8	7	223CD male	5	6
44 B male	5	8	62 D male	5	5
176 B female	7	7	234 D female	4	6
176 B male*	5	4	234 D male*	5	2
176 B female*	3	7	234 D female*	3	3
176 B male*	4	8	234 D female*	2	1
219 B female	6	11	249 D female	1	2
219 B male*	3	6	249 D male*	1	2
219 B female*	6	5	249 D female*	3	4
219 B female*	7	9	249 D male*	3	2
170 B female	11	11	238 D female	11	8
170 B male*	5	13	238 D male*	1	4
170 B male*	4	3	238 D male*	2	2
170 B female*	5	2	238 D female*	3	2

*denotes pups

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