

ASSOCIATION BETWEEN HIGH PINEAL FLUORIDE CONTENT AND PINEAL CALCIFICATION IN A LOW FLUORIDE AREA

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ABSTRACT: Pineal calcification is considered to be the cause of age-related reduction of melatonin. The fluoride ion (F) has been implicated with pineal gland calcification as it is readily incorporated into hydroxyapatite, forming the more stable fluoroapatite. This study collected pineal glands, bone from the 6th rib and humerus, and the trapezius muscle from 42 cadavers from a low-F consumption area in the northeast of Thailand and analyzed the specimens for calcium and F by atomic absorption spectroscopy and a F electrode, respectively. The median F concentration in the pineal gland was 0.132, with an interquartile range of 2.2 (range 0.016–831), mg F/kg gland wet weight. The average F levels in the bone from the 6th rib and humerus were 104.1±31.5 and 85.7±20.1 mg F/kg wet weight, respectively. The F levels in the pineal glands were lower than in the bones (humerus and 6th rib) ($p<0.001$). The calcium concentration in the pineal gland was 77,626±45,600 mg Ca/kg pineal wet weight. No correlation between the F and the calcium contents was found overall for all the pineal glands ($r^2=0.287$, $p=0.077$, $N=53$), but for pineal glands that had a F concentration of more than 50 mg F/kg pineal gland wet weight a strong positive correlation was found between the F and calcium contents ($r^2=0.915$, $p<0.001$, $N=5$). This result implies that a high pineal F content may be associated with increased pineal calcification.

Keywords: Calcification; Fluoride; Pineal fluoride content; Melatonin; Pineal gland.

INTRODUCTION

The pineal gland is a small gland located in the mid-line of the human brain and outside of the blood brain barrier (BBB). It has the second richest capillary network after the kidney.¹ Its main function is to produce melatonin which acts as a negative feedback to the biological clock, the suprachiasmatic nucleus (SCN), that regulates the circadian rhythm of the body. The SCN sends signals to all the organs synchronizing the day-night cycle which leads them to function at the proper time. This can be seen, for example, with sleep, lowering of the body temperature, and lowering of the blood pressure at night time.^{2,3} Melatonin production decreases with age,^{4,5} and may result in dysfunction and pathological outcomes such as chronic primary insomnia,^{6,7} hemorrhagic/ischemic stroke,⁸ schizophrenia,^{9, 10} cancer,¹¹⁻¹³ and Alzheimer's disease.^{14, 15}

Melatonin levels in humans have been shown to be associated with the amount of uncalcified solid pineal tissue, measured by magnetic resonance¹⁶ and computed tomography.¹⁷ Thus, pineal calcification is suspected to be one of the main reasons for melatonin reduction.

Pineal tissue calcification is composed of hydroxyapatite (HA) which is very similar to that found in bones or teeth.¹⁸⁻²¹ Factors that have been considered as

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related to increased pineal calcification are increasing age, male gender, high altitude, and increased intensity of sunlight exposure.^{22,23}

Ordinarily, the amount of the fluoride ion (F) in the brain is very low as F does not readily cross the BBB.²⁴ However the pineal gland is outside the BBB and is thus directly exposed to systemic F. F can readily replace hydroxide and carbonate groups of HA producing fluoroapatite (FA), which is less soluble and more thermodynamically stable and kinetically favored than HA.²⁵ To date, the study of Luke is the only one to investigate the correlation between F and calcium in the pineal gland.²⁶ She found a positive correlation ($r=0.73$, $p<0.02$).²⁶ However, with a small sample (N=11), no correlation was found between F in the pineal gland and bone, a marker for chronic F exposure, although the type of the bone used was not controlled from subject to subject.²⁶

Thus, the relationship between chronic F exposure and F in the pineal gland is of continued interest. Reported markers for chronic F exposure are bones and tooth dentin.²⁷ Nails^{28,29} and blood, bone surface, saliva, milk, sweat and urine³⁰ are reported to be only short term markers, and only correlate with acute exposure.

Zipkin et al, found that flat bone (vertebra, rib bone, and iliac crest) F concentrations were linearly related to water F concentration and found no difference between different types of flat bone.³¹ There are no studies to date that have compared the F content between flat bones and long bones. The dentin of primary molar teeth has also been suggested to provide a satisfactory biomarker for chronic F exposure.³²

Thus, this study measured and correlated the F content in the pineal glands, the trapezius muscle, and the bone of cadavers exposed to low natural levels of F, evaluated from the F levels of the local drinking water. The calcium and F levels in the pineal glands were compared to the F levels of the cancellous bone of the 6th rib, a flat bone, and the cortical bone of the humerus, a long bone.³¹ There are no studies to date that have compared the F content between flat bones and long bones.

MATERIALS AND METHODS

All the tissue and bone samples were collected from cadavers bequested to the Department of Anatomy, Faculty of Medicine, Khon Kaen University. All of the subjects had lived in the northeast part of Thailand before death. The pineal glands, the trapezius muscle, 6th rib bone (cancellous bone), and humerus bone (cortical bone) were collected from 42 cadavers, with a mean age is 67 ± 15.2 yr (range 33–91 yr). Information on gender, cause of death, underlying diseases, and domicile data were available. After harvesting from the cadavers, the pineal glands and trapezius muscles were immediately preserved in buffered 10% formalin. The study was approved by the Khon Kaen University Ethics Committee for Human Research.

Sample preparation of pineal gland: Pineal glands were dried on tissue paper at room temperature for 1 hr, weighed with a Mettler Toledo AT205 balance, crushed

with 1 mL of ultra pure water in an agate mortar and pestle, sonicated for 10 min, and then mixed with 2 μL of 6M H_2SO_4 .

Sample preparation of bone (6th rib and humerus): Bone samples were scraped with a surgical blade to remove any remaining tissue, calcined in a muffle furnace at 550–600°C for 8 hr, and then crushed with an agate mortar and pestle. About 500 mg of bone ash was mixed with 3 mL of 2M HClO_4 prior to the atomic absorption analysis.

Sample preparation of trapezius muscle: The muscle tissue was dried on tissue paper at room temperature for 1 hr, and then about 100 mg of dried muscle was weighed with a Mettler Toledo AT205 balance, crushed with 1 mL of ultra pure water in an agate mortar and pestle, and sonicated for 10 min. Two μL of 6M H_2SO_4 were then added.

Fluoride ion measurement: F was extracted from all the prepared samples by the HMDS-facilitated diffusion method³³ in Falcon 351007 coated Petri dishes. F was measured by a fluoride ion-specific electrode (Orion Model EA 940 Multi-Channel Meter with an Orion 9409BN electrode). We used the two electrode system, inverting the reference electrode to allow 50 μL of each sample to be pipetted onto the top of the head of the inverted reference electrode, then setting the fluoride electrode above, providing a thin film of sample solution between the two electrodes (Figure 1).

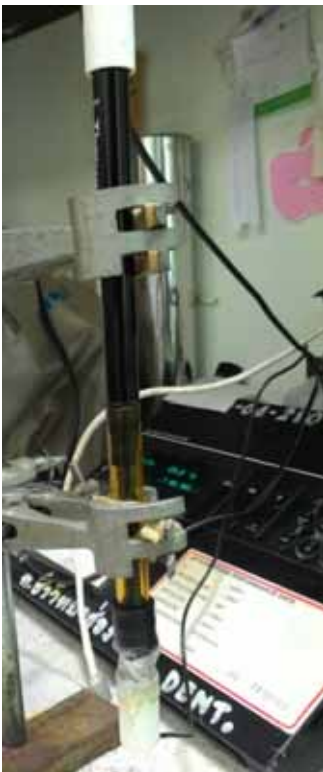


Figure 1. Electrode positions for the modified protocol, with the fluoride electrode above, and the calomel reference electrode below.

All films were reapplied three times. This allowed measurement of only 50 μL of sample solution, removing the necessity of diluting to 2–3 mL for side by side electrodes, and thus increasing the sensitivity more than 100-fold compared with the recommended 5 mL for dual electrode systems. The F concentration of samples was determined from the mV readings on standard curves using F concentrations of 100 ppm, 10 ppm, 1 ppm, 0.1 ppm, and 0.01 ppm (100.0 \pm 0.5 ppm Orion fluoride standard, cat. no. 940907). Calibration curves were measured before and after the samples to ensure there was no drift in measurements. Total ionic strength adjustment buffer (TISAB) was not used as there were no differences in ionic strengths between the samples.

Calcium measurement: Calcium analysis followed the procedure of Luke.²⁶ The remaining pineal gland solution after fluoride extraction was filtered through Whatman No.1 filter paper to put into a glass tube, then 1 mL of concentrated HNO_3 was added. The tubes were warmed to 50°C and kept at 50°C for 30 min in a fume cupboard. The procedure was repeated using 1 mL 60% HClO_4 . Two mL of ultra pure water was then added to each tube and the volumes were measured. To suppress ionization, 0.1% lanthanum, as lanthanum chloride, was added. The calcium concentration was evaluated by atomic absorption spectroscopy (AAAnalyst 100 PerkinElmer instrument) using an oxidizing (lean, blue) air-acetylene flame with a 0.7 nm slit width and detection at 422.7 nm. The calibration curves were linear over the range 0–200 ppm Ca. Phosphate was tested and was not found to interfere with the calcium determination under the conditions used.

Statistical analysis: The F concentration in the pineal glands was found not to have a normal distribution so is reported as the median and interquartile range. The calcium concentration in the pineal glands, and the F concentrations in bones and muscles are shown as means \pm standard deviation (SD). Where normally distributed, differences between groups were evaluated by the unpaired Student's t-test and statistical significance was taken as $p < 0.05$. The Spearman's correlation was used to evaluate the correlation between the F and calcium concentrations in the pineal glands, the F concentration in the pineal glands and bone, and the F concentration in the pineal glands and the age of the cadavers. Pearson's correlation was used to evaluate the correlation between the calcium concentration in the pineal glands and the ages of the cadavers.

RESULTS

The standard curve for the fluoride electrode was linear over the range 0.01 to 100 ppm F, i.e., over four orders of magnitude. There was excellent agreement between the calibration curves using 10 mL of the fluoride standards with the standard side-by-side electrode configuration, and the modified inverted electrode protocol using only 50 μL (Figure 2). The inter-day and intra-day calibration curve correlations were $r^2 = 0.996$ and $r^2 = 0.991$ respectively.

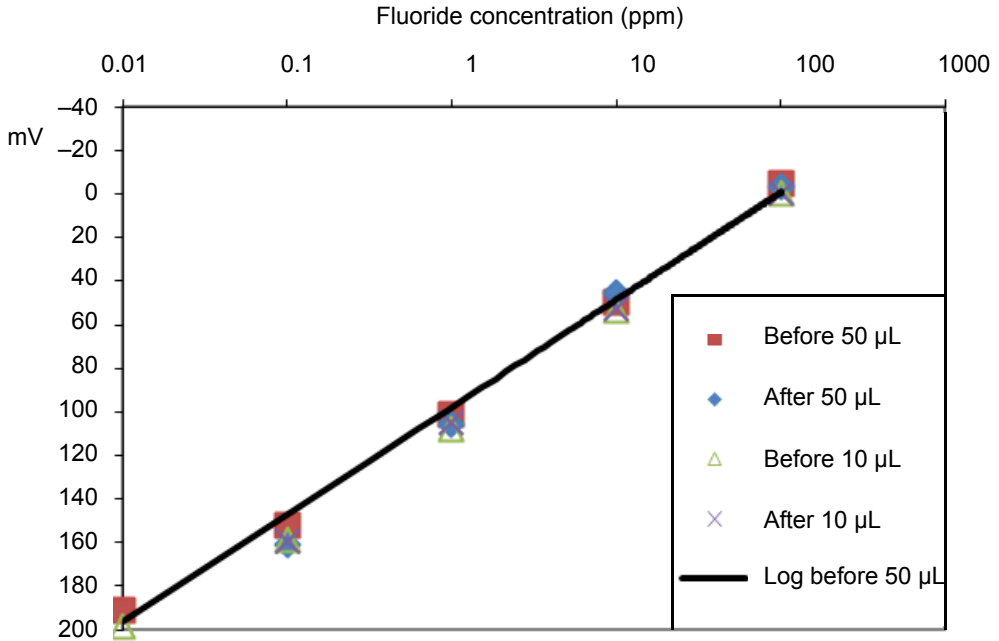


Figure 2. Typical standard curve from the adapted fluoride electrode protocol. Before and after refers to the measurement of standard solutions before and after the measurement of sample solutions, and confirms the stability over the measurement period. $y = -21.41 \ln(x) + 97.76$; $r^2 = 0.996$. The mV are relative. Fluoride electrode calibration done on Sept 30, 2014.

The mean pineal gland weight was 0.039 ± 0.031 g (0.007–0.185 g) (Figure 3).

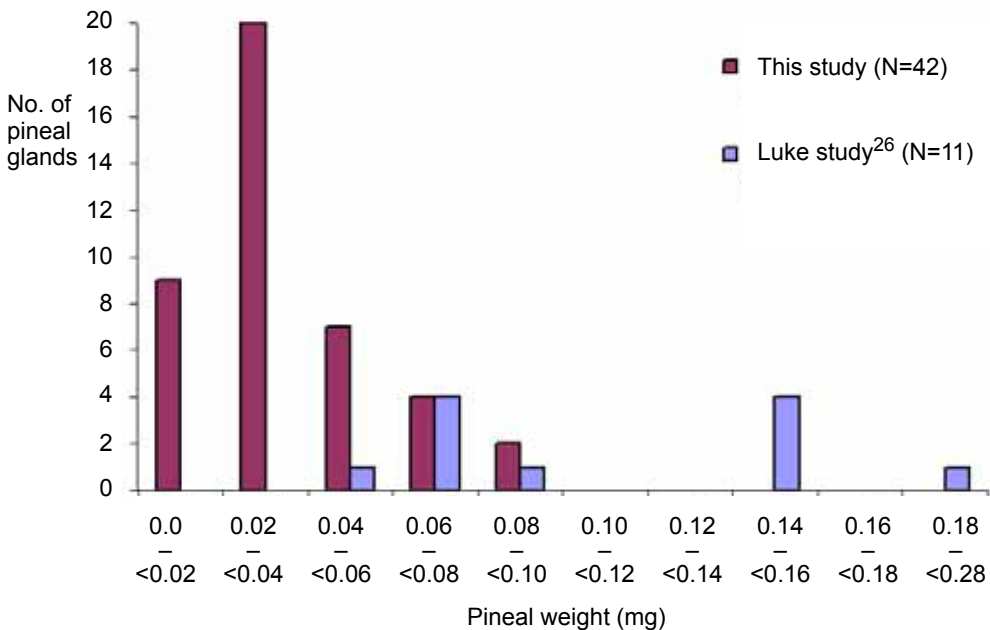


Figure 3. Comparison of the pineal weight (mg) between this study (N=42) and the Luke study (N=11).²⁶

The median fluoride concentration in the pineal glands was 0.132 with an interquartile range of 2.2 (range 0.016–831) mg F/kg pineal gland wet weight (Figure 4).

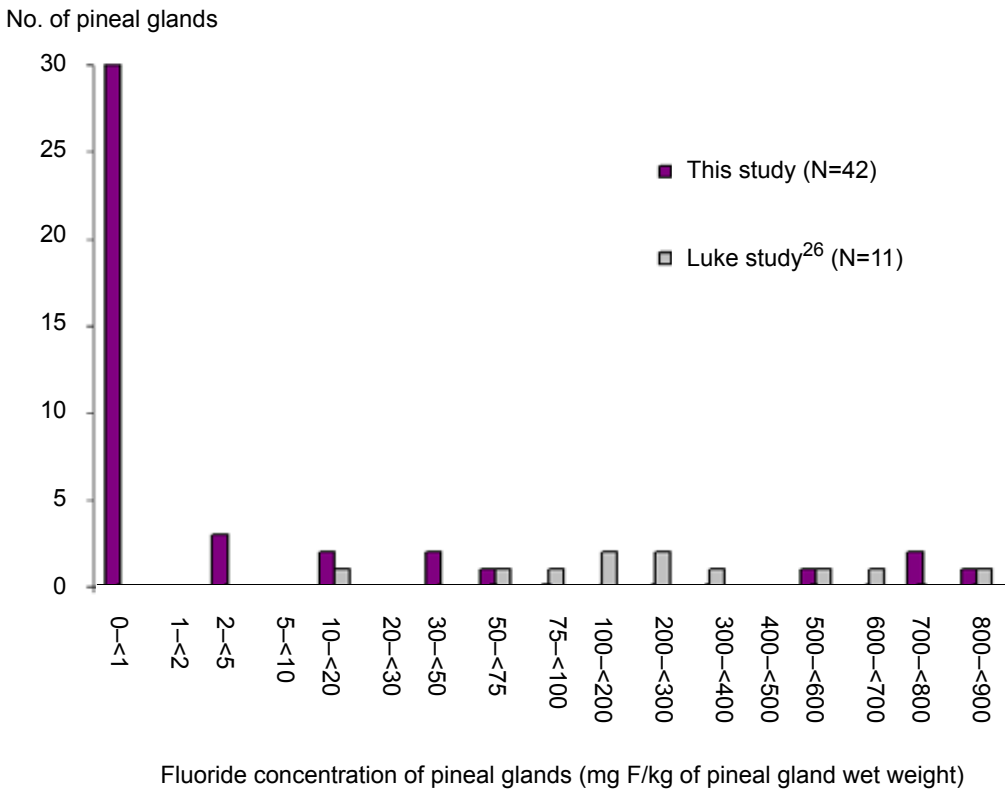


Figure 4. Comparison of the pineal fluoride concentration (mg F/kg of pineal gland wet weight) between this study (N=42) and the Luke study (N=11).²⁶

The mean fluoride concentration in the trapezius muscle was 0.021 ± 0.040 (1.65×10^{-5} –0.245) $\mu\text{g F/kg}$ wet muscle weight. The mean calcium concentration in the pineal gland was $77,626 \pm 45,600$ (range 23,498–213,438) mg Ca/kg pineal gland wet weight.

There was no correlation between the fluoride and calcium contents for all the pineal gland samples (N=42, $r^2=0.287$, $p=0.077$) as most of the glands had a very low fluoride concentration. However, for pineal glands having more than 50 mg fluoride/pineal gland a positive correlation between fluoride and calcium was found (N=5, $r^2=0.915$, $p<0.001$) (Figure 5). No correlation was found between the fluoride concentration in the pineal gland and the age of the cadaver, ($r^2=0.203$, $p=0.192$), but there was a positive correlation between the calcium concentration in the pineal gland and the age of the cadaver ($r^2=0.33$, $p=0.04$).

Fluoride content of pineal gland (μg) for pineal glands with a fluoride concentration of more than 50 mg F/kg pineal gland wet weight

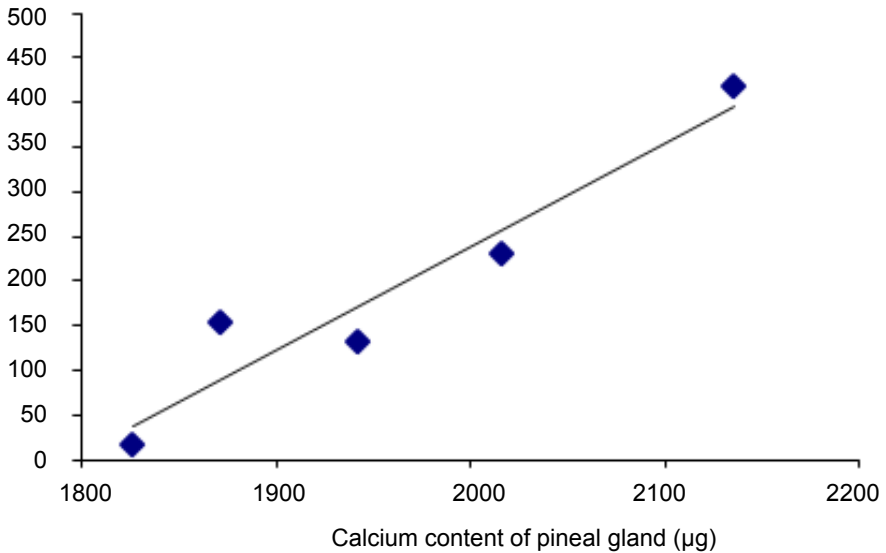


Figure 5. The relationship between the calcium (μg) and the fluoride (μg) contents of the pineal glands with a fluoride concentration of more than 50 mg F/kg pineal gland wet weight. $y = 1.160x - 2081$; $r^2 = 0.915$.

The fluoride concentration in the bones showed a normal distribution, with the mean fluoride levels in the 6th rib and humerus bones being 104.1 ± 31.5 (58.9–215.2) and 85.7 ± 20.1 (40.1–131.9) mg F/kg dry ashed bone weight, respectively (Figure 6). These values were significantly different by the paired t-test ($p=0.002$).

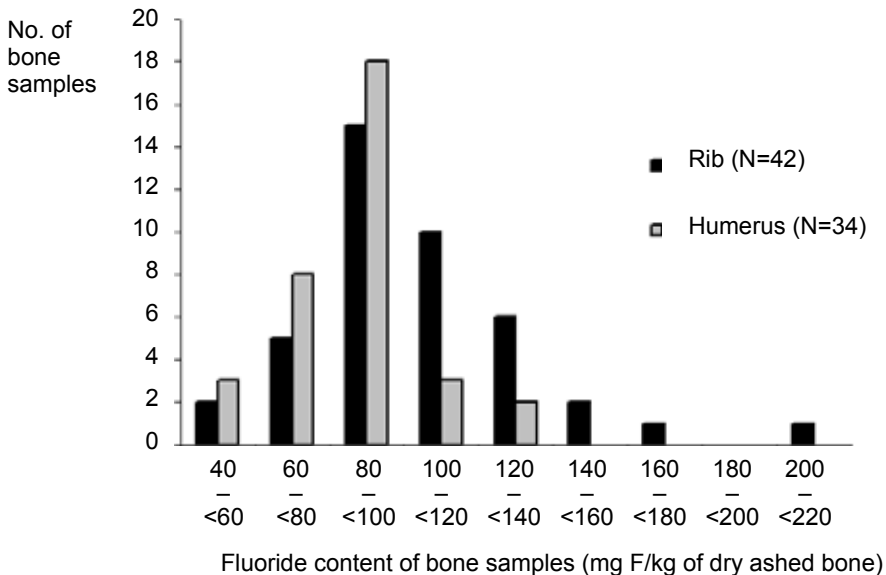


Figure 6. Distribution of fluoride in the dry ashed bone samples from the 6th rib (N=42) and the humerus (N=34).

The fluoride in the trapezius muscle (N=42) was significantly lower than in the pineal glands ($p<0.001$) and the bones (6th rib and humerus) ($p<0.001$) (Figure 7).

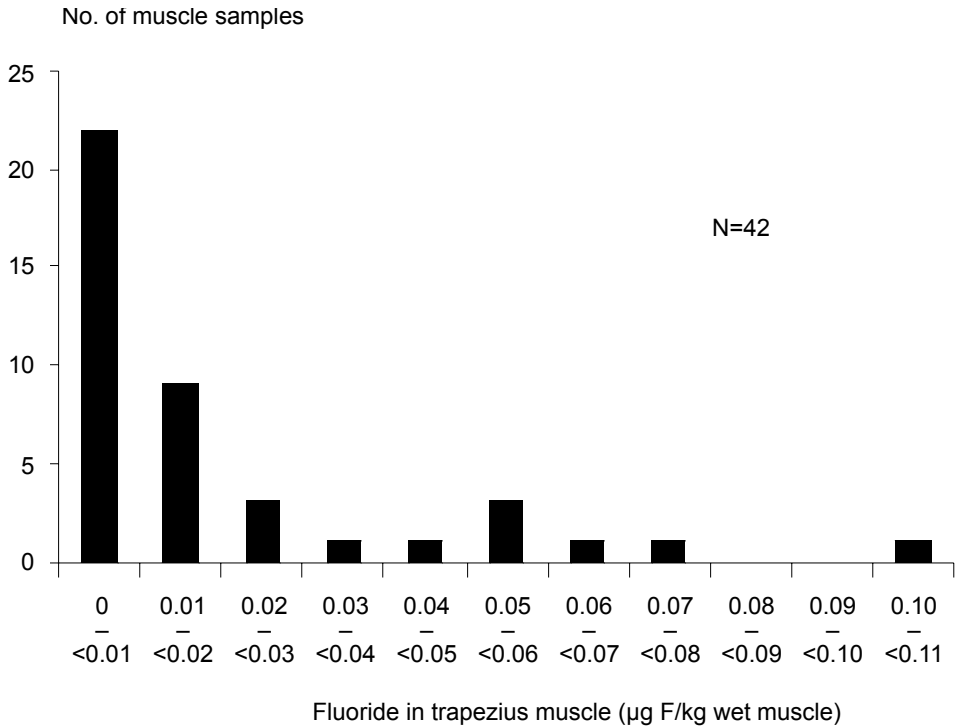


Figure 7. Distribution of fluoride ($\mu\text{g F/kg wet muscle}$) in the wet trapezius muscle samples (N=42).

No correlation was found between the fluoride concentration in the pineal gland and the fluoride concentration in bone. The F level in the pineal glands (median F concentration in the pineal glands: 0.132 with an interquartile range of 2.2 [range 0.016–831] mg F/kg pineal gland wet weight) was lower than in the bones (mean F levels in the 6th rib and humerus bones: 104.1 ± 31.5 [range 58.9–215.2] and 85.7 ± 20.1 [range 40.1–131.9] mg F/kg dry ashed bone weight, respectively) ($p<0.001$).

The percentage calcification of the pineal glands ranged from 5.9 to 53.6% (mean 19.5 ± 11.4) based on the calcium content and the weight of the glands. This is somewhat higher than that reported by Luke of 0.4 to 11.7% (mean 3.9 ± 3.6), probably due to the smaller size of the pineal glands (0.007–0.185 g, mean 0.039 ± 0.031 g) in this study, than in Luke's study (0.056–0.198 g, mean 0.112 ± 0.052 g).²⁶ Another study also reported pineal weights of 0.099 ± 0.056 g and 0.091 ± 0.041 g in 168 elderly male and females, respectively.³⁴

DISCUSSION

We adapted the fluoride electrode method because the final volume of the extracted solution from the HMDS-facilitated diffusion method³³ was lower than the minimum volume that can be measured with a dual electrode system. The standard curve for the validated adapted method had an acceptable correlation ($r^2=0.996$ intra-day, 0.991 inter-day). In contrast to Luke's study,²⁶ when all the pineal glands were considered we found no correlation between the fluoride and the calcium concentrations in the pineal gland ($r^2=0.287$, $p=0.077$).

This is only the second reported study to evaluate the fluoride concentration in the pineal gland, and the first to systematically compare this with the fluoride concentration in bone. The mean fluoride amount in the pineal glands in this study was much lower at 75.5 ± 228 (range 0–831) mg F/kg pineal gland wet weight) (Figure 4), compared to Luke's study²⁶ which reported 314.5 ± 374.3 (range 14–875) mg F/kg pineal gland wet weight, although the range was similar.

The mean fluoride levels in the 6th rib and humerus bones, 104.1 ± 31.5 (58.9–215.2) and 85.7 ± 20.1 (40.1–131.9) mg F/kg dry ashed bone weight, respectively, in this study were much lower compared to Luke's study²⁶ which reported $2,037\pm 1,095$ (range 838–3,711) mg F/kg bone ash weight.

The mean fluoride in trapezius muscle was about five times lower at 0.021 ± 0.040 (range 1.65×10^{-5} to 0.245) μg F/kg muscle wet weight compared to Luke's study²⁶ which reported 0.5 ± 0.4 (range 0.2–1.5) mg F/kg muscle wet weight.

The mean weight of calcium in the pineal glands in this study was $77,626\pm 45,600$ (range 23,498–213,438) mg Ca/kg wet pineal gland weight, compared to Luke's study²⁶ which reported $16,000\pm 11,070$ mg (range 4,600–37,250) Ca/kg wet pineal gland weight.

Very low concentrations of F were found in muscle, as expected, as fluoride does not accumulate in muscle tissue. Fluoride could be measured at very low concentrations in bone and muscle ($<1\mu\text{g}$ F/kg wet weight) due the availability of a large quantity of sample compared to samples of dentin and of pineal gland.

The calcification levels, i.e., the weight of HA based on the Ca content as a proportion of the total pineal gland weight, were similar to those reported by other workers using microanalytical techniques, for example 38% Ca in the center of concretions using an electron microprobe,³⁵ 28.6–38.2% Ca by EDX, 38.5% Ca by EDS,³⁶ and 20–32% from element maps of whole pineal glands by electron microprobe.³⁷ Similar calcium levels for enamel (37.7%), dentine (36.41%), and bone (35.8%) have been reported.³⁸

Based on the calcium:fluoride ratios in the pineal glands, we were able to estimate the percentage replacement of OH in HA $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$ by fluoride as 0–29% (mean $4.1\pm 8.1\%$).

The upper level (29%) is the same as that found for pure FA that contains 3.7% fluoride by weight, i.e., about one third of the OH ions can be replaced by

fluoride.³⁹ The levels of fluoride that would be required to produce pure FA *in vivo* would be highly toxic (above 5 mg/kg body weight, i.e., the dose for acute toxicity). The mechanism of fluoride incorporation into pineal calcification therefore implies a slow substitution from lower fluoride plasma concentrations via chronic exposure.

The northeast part of Thailand where the subjects lived has very low fluoride levels in the natural drinking water of approximately 0.03–0.07 ppm.^{40,41} There is no government or commercial fluoridation of water supplies in Thailand, and although some commercially bottled water is fluoridated (0.03 to 0.72 ppm, mean 0.17 ± 0.16 ppm), only three brands contain appreciable amounts of fluoride.⁴² Two of these are imported and not widely consumed.⁴² The consumption of commercial bottled water is low among the subject population. High levels of naturally occurring fluoride in water are found in some localized regions in the country, giving rise to fluorosis, but these are in the far north, far distant from the area where the subjects dwelled. From the study of Zipkin et al.³¹ who correlated the bone fluoride with the fluoride intake from water, we estimate the content of fluoride in the water consumed by the subjects in this study was lower than 0.1 ppm, in agreement with the data presented on the water analysis.^{40,41} Furthermore, the use of fluoride varnish by dental professionals, a potential extra-dietary source of fluoride is relatively recent and limited to children. Our subjects were elderly and would not have been exposed to such treatment. Our subjects would also not have been likely to have received significant fluoride from toothpaste, as fluoridized toothpaste is a relatively recent development. Furthermore, adults are not expected to swallow toothpaste, as children may do. We can thus confirm that the subjects of this study had a low fluoride consumption.

Luke's study was conducted in the mid-1990's at Surrey University. Up to 1982, the only major cities to be fluoridated in the United Kingdom were Birmingham and Newcastle. Many areas in England have a significant natural fluoride content in the drinking water (0.5–0.9 ppm), including Norwich, Ipswich, Cambridge, Hartlepool, Slough, Colchester, and other sites particularly in the counties of Essex, Norfolk, Suffolk, and in North East London, all areas near Luke's study site.⁴³ Other areas are reported to have less than 0.5 ppm fluoride in water.⁴⁴

Of significant note is that although the fluoride concentrations in the pineal were mostly very low with only a few subjects exhibiting high fluoride concentrations, in bone, the fluoride concentration was 60–215 mg/kg and normally distributed, with no subjects showing either very low or very high levels (Figure 6). This finding for bone is consistent with chronic exposure to low levels of fluoride from naturally low levels in water and food.³¹

A previous study reported the fluoride level in bone varying between 1295 to 5745 (mean 2824) mg F/kg for the iliac crest, and increasing with age.⁴⁵ However neither the rib bone or the humerus bone fluoride content correlated with age in the present study.

By contrast, the fluoride levels in the trapezium muscle were extremely low. This is consistent with other studies, and confirms that muscle does not accumulate fluoride. It also gives us confidence in measuring fluoride at low levels (between 0.01 and 1 ppm) in solutions from tissue samples.

High levels of fluoride have been reported in the serum of patients undergoing haemodialysis and continuous ambulatory peritoneal dialysis.⁴⁶ However none of the subjects in our study were reported to have had end-stage renal disease or were undergoing dialysis.

Given the expected low levels of fluoride exposure of the subjects in this study, based on the water consumption, the lack of water fluoridation, and the low concentrations of fluoride found in bone, it is difficult to explain why a few individuals exhibited much higher concentrations of fluoride in their pineal glands (five pineal glands had more than 50 mg F/kg of pineal gland wet weight, Figure 4). However, the strong correlation between the pineal calcium and fluoride contents in these cases implies that pineal fluoride incorporation is related to calcification (Figure 5). No examples of higher fluoride concentrations were found at lower levels of calcification. It has been suggested that calcification is not a natural process, but a pathological process, perhaps as a result of lack of calcium control in the tissue and surrounding fluid,^{47,48} although the incorporation of fluoride itself is likely to be a passive process within the calcification process.

CONCLUSIONS

This is only the second study to investigate fluoride in calcifications of human pineal gland. Although it is not possible to conclude that low fluoride exposure can lead to increased or more rapid calcification in the pineal gland, higher pineal fluoride levels appear to be associated with a higher degree of pineal calcification in agreement with Luke's study.²⁶ Fluoride was found to deposit more in flat bone than in long bone.

Further study of the pineal glands of subjects with a high fluoride content should be conducted to further the understanding of the relationship between fluoride intake and pineal calcification in humans.

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