Clin Oral Invest (2016) 20:2361–2370
DOI 10.1007/s00784-016-1727-1

ORIGINAL ARTICLE

# Deterioration of teeth and alveolar bone loss due to chronic environmental high-level fluoride and low calcium exposure

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Received: 3 April 2015 / Accepted: 18 January 2016 / Published online: 28 January 2016


**Abstract**
*Objectives* Health risks due to chronic exposure to highly fluoridated groundwater could be underestimated because fluoride might not only influence the teeth in an aesthetic manner but also seems to led to dentoalveolar structure changes. Therefore, we studied the tooth and alveolar bone structures of Dorper sheep chronically exposed to very highly fluoridated and low calcium groundwater in the Kalahari Desert in comparison to controls consuming groundwater with low fluoride and normal calcium levels within the World Health Organization (WHO) recommended range.
*Materials and methods* Two flocks of Dorper ewes in Namibia were studied. Chemical analyses of water, blood and urine were performed. Mineralized tissue investigations included radiography, HR-pQCT analyses, histomorphometry, energy-dispersive X-ray spectroscopy and X-ray diffraction-analyses.
*Results* Fluoride levels were significantly elevated in water, blood and urine samples in the Kalahari group compared to the low fluoride control samples. In addition to high fluoride, low calcium levels were detected in the Kalahari water. Tooth height and mandibular bone quality were significantly decreased in sheep, exposed to very high levels of fluoride and low levels of calcium in drinking water. Particularly, bone volume and cortical thickness of the mandibular bone were significantly reduced in these sheep.
*Conclusions* The current study suggests that chronic environmental fluoride exposure with levels above the recommended limits in combination with low calcium uptake can cause significant attrition of teeth and a significant impaired mandibular bone quality.
*Clinical relevance* In the presence of high fluoride and low calcium-associated dental changes, deterioration of the mandibular bone and a potential alveolar bone loss needs to be considered regardless whether other signs of systemic skeletal fluorosis are observed or not.

**Keywords** Fluoride · Fluorosis · Sheep · Histomorphometry · Alveolar bone · HR-pQCT

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## Introduction

Fluoride has been known for decades to be protective to teeth and substantially reduce tooth decay [1]. Fluoride is incorporated into hydroxyapatite crystals in collagen bundles, particularly in bone and teeth, and confers increased resistance to acid dissolution and/or refined carbohydrates, e.g. due to food

intake [2]. Additionally, fluoride has been used since the 1980s for the treatment of bone disorders, particularly osteoporosis. Its use was significantly reduced with the introduction of new osteoporosis-specific pharmaceutical products over the last decades [3–5].

The recommended fluoride content in water is 0.7–1.2 mg/l [6, 7]. If concentrations exceed these amounts (2–8 mg/l), dental fluorosis, i.e. discoloration and marked wear on biting surfaces, may result [8]. With even higher concentrations of 8–20 mg/l of fluoride, there may even be negative effects on bone mineral tissue, causing osteosclerosis or even leading to crippling skeletal fluorosis [6, 9]. Skeletal fluorosis is a condition hallmarked by osteosclerosis, reduced bone quality and ligament calcifications; it can however also be accompanied by low bone mass [10, 11].

Previous studies demonstrated the influence of fluoride in humans regarding dental caries [12–14]. However, these observations were made (i) with moderately elevated amounts of fluoride, (ii) mostly focused on caries incidence and (iii) did not include the alveolar bone. Since the primary focus of these studies was to analyse the incidence of dental fluorosis in relation to fluoride content in the drinking water, effects of fluoride on the bone are missing.

Dorper Sheep from the southwestern region of Namibia in the Kalahari Desert, living on very highly fluoridated groundwater with low levels of calcium, were supplied for this study. These sheep showed much greater tooth deterioration than sheep of the same age from other parts of Namibia exposed to recommended fluoride and calcium levels in the groundwater, as reported by the local farmers. Hence, the purpose of the current study was to identify if and how the teeth and alveolar bone of sheep are affected by long-term environmental exposure to high fluoride and low calcium doses. An analysis of the water was performed to determine the fluoride concentration and its general electrolyte composition. Further analyses included radiographic imaging and structural evaluations in order to detect the possible influence of fluoride in other mineralized tissues associated with the teeth. These analyses should be considered, as many people are exposed to fluoridated water above recommended levels and could also be affected.

## Materials and methods

### Human subjects

This study was carried out according to existing rules and regulations of the University Medical Center Hamburg-Eppendorf and is in line with the "Hamburg Hospital Law (HmbKHG) April 17th, 1991: Patient Security §12". Informed consent was obtained from all subjects and/or their parents.

Simple dental examinations were carried out with a plane dental mirror and an artificial light. All surfaces (buccal, lingual and in posterior teeth also occlusal) of each tooth were examined for signs of dental fluorosis. Further physical examinations were not carried out. The examinations were performed in all the farmers on both farms.

### Animals

The Dorper sheep was originally bred to live and produce under arid conditions. It was developed in South Africa in the 1930s, where it is currently the second largest breed. The first Dorper sheep were brought to Namibia in 1964. Namibia is a dry desert country and is not very suitable for farming or cattle except for sheep. Dorper sheep have a thick skin, which protects them from harsh climatic conditions. In Namibia, Dorpers make up approximately 80 % of all sheep. The Dorper is a free-range sheep that thrives on bushes, shrubs and water [15].

In this study, a total of 10 female Dorper sheep from two different farms in Namibia were studied. Five sheep at the age of 6–7 years were randomly selected from each farm. Five sheep at the age of 6–7 years were randomly selected from each farm. Five sheep were from a farm located in the southwestern Kalahari Desert area with high fluoride concentrations (9.8 ± 0.3 mg/l) in the water, which results in observable tooth abrasion in the sheep. The control flock (low fluoride group; $n = 5$) was from a farm outside the Kalahari with recommended fluoride levels (0.6 ± 0.3 mg/l) in the groundwater. Both free-range Dorper sheep flocks lived under the same arid conditions and had equal pasture (shrubs, bushes and water from drinking sources) limited by the desert climate and the hard and rough surfaces.

Water samples were collected from livestock drinking sources at both farms and analysed with specific attention to the fluoride content. Sampling of blood, urine, jaws and bones was carried out immediately after sacrifice.

The water and urine samples were stored at −80 °C until assayed. Blood samples were centrifuged at 4 °C to obtain plasma, which was stored at −80 °C until further assays were performed. The bones and teeth were fixed in 3.7 % PBS-buffered formaldehyde for 3 days at −4 °C until they were transferred into 70 % ethanol and stored for further assays.

This study was carried out following the current rules and criteria of the local ethics committee (LEC). All samples were collected during the standard slaughtering process executed by trained farm employees.

### Fluoride analyses

The groundwater analyses were executed at the IWW Water Research Institute (Rheinisch-Westfaelisches Institut für Wasser, Muelheim an der Ruhr, Germany). The fluoride analyses were performed by the determination of dissolved anions by liquid chromatography of ions by following the standard

DIN EN ISO 10304-1. The water samples were analysed for nitrate (mg/l), fluoride (mg/l), chloride (mg/l), sulfate (mg/l), sodium (mg/l), potassium (mg/l), calcium (mg/l), and magnesium (mg/l). Blood and urine samples were analysed potentiometrically using an ion-selective electrode for fluoride in the laboratories of Medizinisches Labor Bremen (Medizinisches Labor Bremen, Bremen, Germany, DIN EN ISO 17025 and 15,189). Samples were analysed in a blinded fashion. Both laboratories are QC certified. The quality control is carried out according to the guidelines of the German Medical Association (Richtlinien der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen). Additional equivalent measurements are therefore not required and have not been carried out at our samples. To analyse the distribution of fluoride within the mineralized tissues Energy-dispersive X-ray spectroscopy (EDX) was performed on the specimens (see the "Energy-dispersive X-ray spectroscopy, quantitative backscattered electron imaging and X-ray diffraction" section).

### Contact radiographs, high-resolution peripheral quantitative computer tomography and ground sections

The mandibles and teeth of all sheep were analysed by contact radiography using a Faxitron X-ray cabinet (Faxitron X-ray Corp., Wheeling, IL, USA). Measurements of mandible microarchitecture were performed at the angle of the mandible.

Structural bone parameters (C.Th: cortical thickness, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular spacing, BV/TV: bone volume per tissue volume) were measured to assess bone microarchitecture changes due to different flouride levels. This enables microarchitectural measurements by bone histomorphometry with the use of established algorithms, as defined by the ASBMR Histomorphometry Nomenclature Committee [16], which can assess 2D various characteristics of the trabeculae, such as thickness and connectivity. Structural assessments and 3D visualization of bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular spacing (Tb.Sp), trabecular thickness (Tb.Th) and cortical thickness (Ct.Th) were performed with a high-resolution peripheral quantitative computed tomography system (HR-pQCT, Xtreme-CT®, Scanco Medical, Bruettisellen, Switzerland). Therefore, the mandibles were scanned (60 kV/900 μA) at a resolution of 80 μm. Secondly, the generated raw data were manually segmented for analyses with the μCT Evaluation Program Version 6.0 (Scanco Medical, Bruettisellen, Switzerland). Finally, the segmented data were imported and displayed in μCT Ray Version 3.8 for visualization (Scanco Medical, Bruettisellen, Switzerland).

Thereafter, the ground sections were cut sagittally in the mandibular pre-molar and molar region. The specimens were embedded in a methyl methacrylate-based resin (Technovit 7200 VLC, Heraeus Kulzer GmbH, Wehrheim, Germany) and ground to 50 μm as previously described [17].

### Energy-dispersive X-ray spectroscopy, quantitative backscattered electron imaging and X-ray diffraction

Energy-dispersive X-ray spectroscopy (EDX) was performed on the incisor region to analyse the distribution of fluoride within the mineralized tissues, where one cross-section was used per individual. Per sample, three regions of interest (ROIs) with a size of 100 μm × 40 μm were selected for EDX analysis. The ROIs consisted exclusively of mineralized tooth/bone volume without pores or voids. The scanning microscope was operated at 20 kV, 580 pA, and a working distance of 24 mm (EDAX/DX-4 Detector, EDAX Inc., Mahwah, NJ, USA). The elemental peak reflecting the fluoride content was evaluated in weight percent (wt%) via the EDX-ZAF software provided by the manufacturer (Version 4, EDAX Inc., Mahwah, NJ, U.S.A).

Quantitative backscattered electron imaging (qBEI) was performed on the same specimens to quantify the calcium concentration within the mineralized phase of the enamel, dentin and alveolar (mandibular) bone. The three tissues: tooth enamel and dentin, and alveolar bone, were analysed separately. The methyl methacrylate-embedded biopsies were ground and polished to a coplanar finish and then carbon coated. The scanning electron microscope (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England) was operated at 20 kV, and the electron beam was kept at 580 pA using a Faraday cup (MAC Consultants Ltd., Stanmore, England). The BSE detector (BSE Detector, Type 202, K.E. Developments Ltd., Cambridge, England) was set up at a working distance of 20 mm. Grey levels were obtained (Image J 1.42, National Institutes of Health, Rockville, MD, USA) and transferred to calcium weight percent as described previously [18, 19]. For visualization purposes, the images were pseudo-colored later. The mean calcium content (Ca mean) and the heterogeneity of the calcium distribution (Ca width) were measured, respectively.

X-ray diffraction (XRD) was used to determine the crystal structure of the teeth using Cu Kα radiation ($\lambda = 0.15406$ nm). The acquisitions were carried out at a scanning rate of 0.05°/s in the 2θ range of 20–80° with the beam incident on the enamel side of the tooth. The XRD spectra were acquired from the incisors from three different sheep per group. The spectra were normalized by the highest diffraction peak, and the spectra were averaged for each group. All samples were analysed in a blinded fashion.

### Statistical analysis

Normally distributed data are presented as mean ± standard deviation. Group differences (low fluoride (LF) vs. very high fluoride and low calcium (VHF/LC)) in continuous data were

assessed by two-tailed *t* tests. Differences were defined as significant (*) when $p < 0.05$ and/or (**) $p < 0.01$.

## Results

### Fluoride analyses and phenotypic effects

In addition to changes in sheep teeth, all farmers (ten of ten) living as permanent inhibitants on a farm located in the southwestern Kalahari Desert area with very high fluoride and low calcium concentrations in groundwater show phenotypic signs of dental fluorosis (Fig. 1a). White opaque areas, pitting and brown stains of the teeth were highly visible among these farmers. None of the eleven farmers of the other farm with low fluoride and normal calcium concentrations had signs of dental fluorosis. Groundwater analyses demonstrated very high elevated mean fluoride levels on the Kalahari Desert farm at 9.8 ± 0.3 mg/l of fluoride in comparison to the "control" farm outside the Kalahari with recommended fluoride levels (0.6 ± 0.3 mg/l) ($p < 0.01$; Fig. 1b). Additional chemical analyses of the water revealed low mean magnesium (<5.0 ± 1.2 vs. 31.3 ± 1.5 mg/l) and mean calcium (< 5.0 ± 0.9 vs. 17.6 ± 1.1 mg/l) values in the Kalahari Desert cohort vs. low fluoride/normal calcium controls. Mean sodium (3410.0 ± 173.1 vs. 114.0 ± 5.7 mg/l) and mean chloride (1680.0 ± 130.2 vs. 27.1 ± 2.1 mg/l) were strongly lowered in the very high fluoride area, whereas mean potassium was nearly unchanged (13.7 ± 1.2 vs. 12.9 ± 0.9 mg/l). Analyses of sheep blood and urine confirmed the increased uptake of fluoride from the groundwater in sheep living in the very high fluoride area ($p < 0.01$; Fig. 1b).

### Energy-dispersive X-ray spectroscopy, quantitative backscattered imaging and X-ray diffraction

Tissue incorporation of fluoride was analysed by EDX elemental analysis for fluorine in the three tissues: tooth enamel and dentin, and alveolar bone. EDX revealed significant fluorine peaks in the alveolar bone ($p < 0.01$), as well as the tooth dentin ($p < 0.01$) and enamel ($p < 0.05$) when compared with the low-fluoride group (Fig. 1c). Significant quantitative differences in the mean weight percentage levels of fluorine were recorded in the three analysed tissues, with the highest incorporation identified in the alveolar bone of the very high fluoride-exposed flock versus the low fluoride/normal calcium control group, 45.3 ± 5.5 vs. 15.0 ± 9.0 wt% ($p < 0.01$), respectively (Fig. 1c).

Additionally, qBEI was used to analyse the mineral content of the alveolar bone, as well as the tooth enamel and dentin of the incisors (Fig. 2). The alveolar bone of very high fluoride-exposed ewes demonstrated a significant difference in the mean calcium content, i.e. 22.4 ± 0.4 vs. 19.9 ± 0.6 wt% for the very high fluoride and low fluoride/normal calcium control groups ($p < 0.01$), respectively. The heterogeneity of mineralization, reflected by the mean calcium width, demonstrated significant changes with 3.9 ± 0.1 wt% for the very high fluoride/low calcium group versus 3.6 ± 0.1 wt% in the low fluoride/normal calcium control samples ($p < 0.05$). Tooth mineralization did not differ significantly in either dentin or enamel (Fig. 2c). The generated mineralization profiles (grey value histograms) from quantitative backscattered electron images for each specimen represent the mean calcium weight percent (mean Cawt%) and indicate the average calcium content in the mineralized bone tissue area, while the width calcium value (Δ width Cawt%) reflects the homogeneity of mineralization, i.e. the width of the calcium content distribution in the given bone area (average of all bone packets).

XRD spectra were acquired from the enamel portion of low fluoride controls and very high fluoride-exposed sheep teeth. The averaged spectra are shown in Fig. 2d.

### Contact radiographs and HR-pQCT

Macroscopic deterioration of the molars and pre-molars of sheep form the very high fluoride and low calcium groundwater area was observable by visual inspection and confirmed by imaging techniques (Fig. 3a). The normal dentition in the lower jaw of the sheep is three or four incisors, three premolars and three molars per side. The height of the molar crown was significantly reduced (Fig. 3b) from mean 8.5 ± 5.1 mm in low fluoride/normal calcium controls to mean 1.7 ± 2.4 mm in very high fluoride and low calcium-exposed samples ($p < 0.05$). No significant changes were detected in the root of the teeth. In addition to the significantly reduced tooth height in the very high fluoride and low calcium-exposed sheep, changes in the alveolar bone were observed in contact radiographs, as compared to low fluoride/normal calcium-exposed sheep (Fig. 3a). HR-pQCT scans of the mandibular angle were executed to identify structural changes in the mandible (Fig. 4). The mean cortical bone volume was significantly reduced in the very high fluoride and low calcium group ($p < 0.05$; Fig. 4b). The trabecular number and thickness were measured at 0.27 ± 0.06 per millimeter and 170 ± 21 μm in the very high fluoride and low calcium group versus 0.37 ± 0.13 per millimeter and 215 ± 46 μm in the low fluoride/normal calcium control samples, respectively. Furthermore, a significant decrease in cortical thickness was measured in very high fluoride and low calcium-exposed sheep (Fig. 4c). The cortical thickness of the mandibular angle in very high fluoride and low calcium-exposed animals was nearly 50 % lower than the low fluoride/normal calcium control group, 0.80 ± 0.16 and 1.57 ± 0.34 mm, respectively ($p < 0.01$).

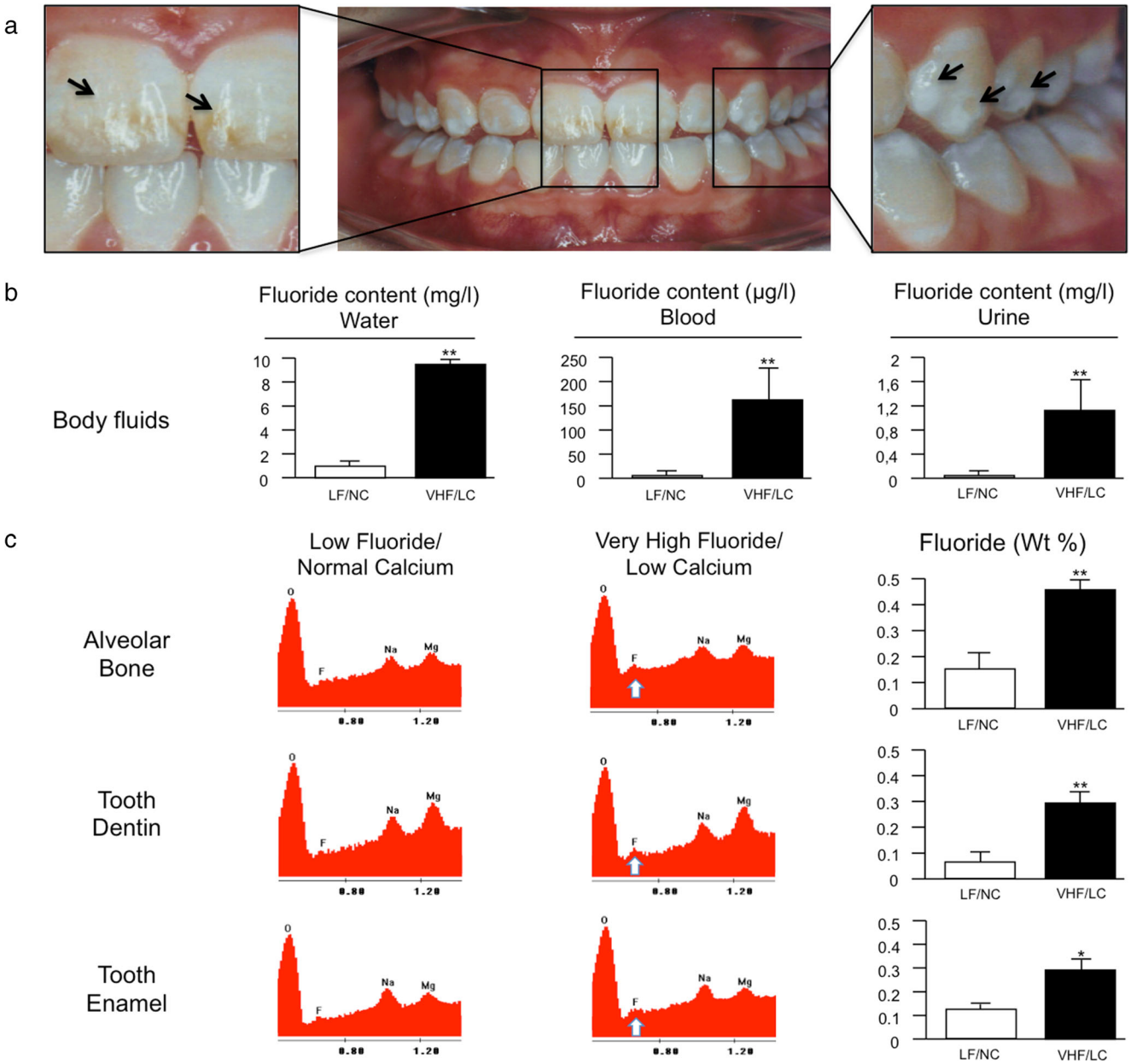
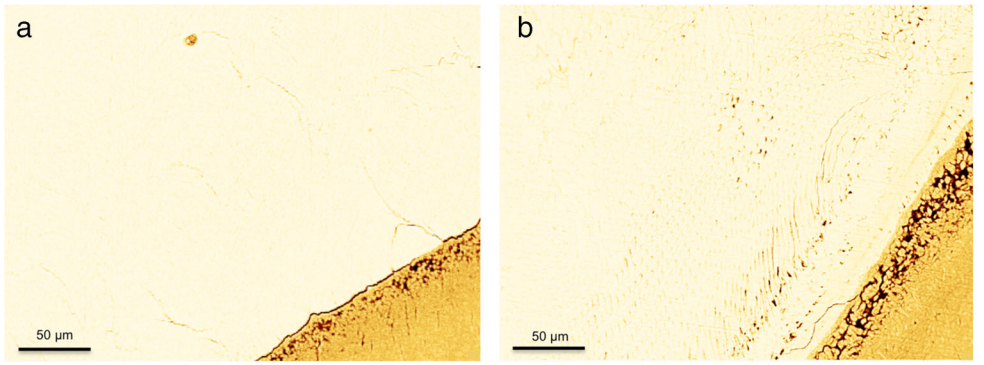



**Fig. 1** Fluoride-affected teeth, body fluids and EDX analyses of fluoride levels. **a** shows a frontal view of fluoride-affected teeth from one of the female farmers at 17 years of age living as a permanent inhabitant on a farm in the Kalahari Desert with chronic (lifelong) exposure to groundwater very high in fluoride and low in calcium. Magnification of the frontal teeth demonstrated small, opaque, paper-white areas and brown discoloration indicated by *arrows*. The other enlargement of the incisors, pre-molars and molar teeth illustrates some more opaque, white spots with some pitting, indicated by *arrows*. **b** shows the fluoride content in the water, blood and urine samples from the Kalahari Desert (very high fluoride and low calcium) and the control farm (low fluoride and normal calcium). The fluoride content was significantly elevated in all three fluid samples of the livestock from the Kalahari Desert. Tissue incorporation of fluoride was confirmed by energy-dispersive spectroscopy (**c**). Fluoride peaks (*white arrows*) were only detectable in chronic very high fluoride exposure samples of the bone of the mandible, tooth dentin and enamel. Fluoride weight percentage levels are demonstrated in the bar graphs. A *single asterisk* indicates significance when $p < 0.05$ or *double asterisks* when $p < 0.01$. *LF/NC* low fluoride and normal calcium, *VHF/LC* very high fluoride and low calcium

## Discussion

Natural exposure to fluoride in groundwater is very common in numerous areas of the world and potentially compromises the health of affected inhabitants [14, 20–24]. The fluoride level measured in the groundwater of the very high fluoride-exposed cohort in the Kalahari Desert was 6.5 times higher than the upper recommended fluoride limit of the World Health Organization (WHO) of 1.5 mg/l [6, 25]. Fluoridated water has fluoride at a level that is effective for preventing cavities, and this can occur naturally or by adding fluoride. However, excessive fluoride exposure is not a rare condition. Kakumano and Rao recently




c

| | | LF/NC | VHF/LC |
|---|---|---|---|
| Tooth Enamel | Ca mean (Wt %) | 36.78 ± 0.28 | 37.11 ± 0.05 |
| | Ca width (Wt %) | 1.38 ± 0.56 | 1.15 ± 0.12 |
| Tooth Dentin | Ca mean (Wt %) | 27.69 ± 0.42 | 27.36 ± 0.81 |
| | Ca width (Wt %) | 2.17 ± 0.34 | 2.07 ± 0.04 |
| Alveolar bone | Ca mean (Wt %) | 19.88 ± 0.57 | 22.39 ± 0.43** |
| | Ca width (Wt %) | 3.66 ± 0.10 | 3.99 ± 0.15* |

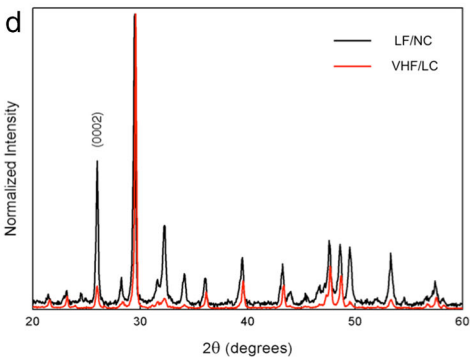
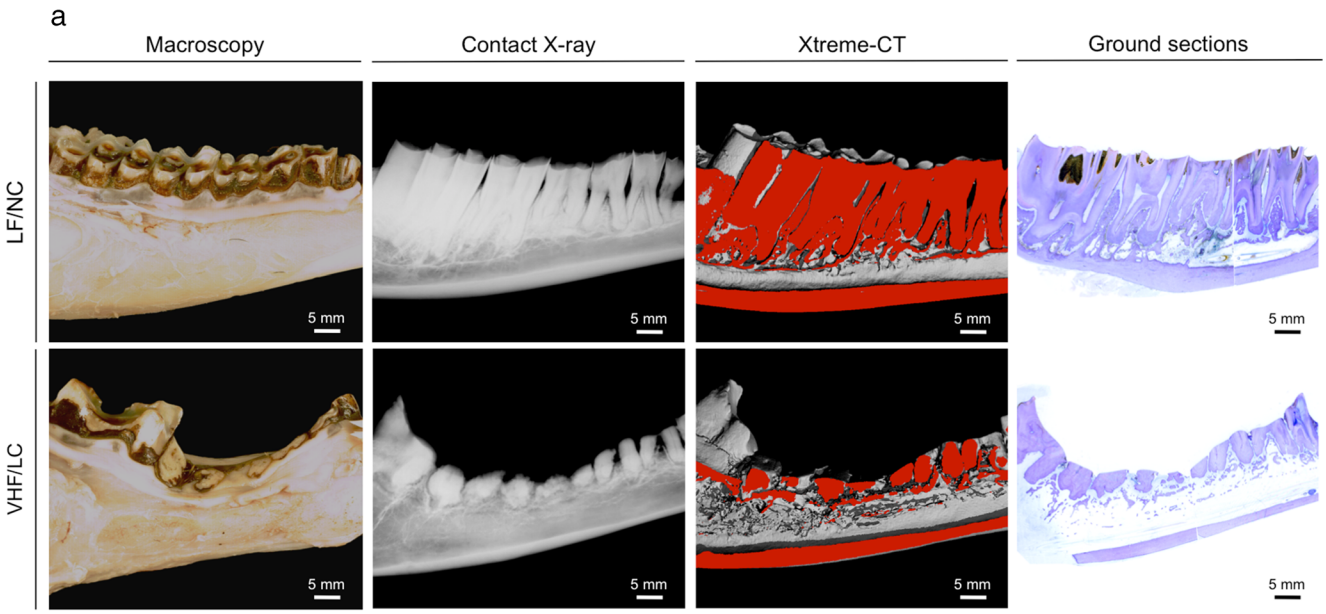



**Fig. 2** qBEI and XRD-results. Pseudo-coloured qBEI images of the enamel and dentin border of low fluoride and normal calcium (**a**) and very high fluoride and low calcium sheep (**b**). Higher mineralization was identified in the enamel as a paler yellow in comparison to dentin with a yellow-orange colour. Bright colours indicate higher mineralization. Mineralization values for enamel, dentin and the alveolar bone demonstrate significant differences in the mean mineralization contents for alveolar bone (**c**). X-ray diffraction (**d**) was performed on the enamel surface of sheep teeth with the X-ray beam incident on the enamel surface of the tooth. The spectra taken for the low fluoride and normal calcium and very high fluoride and low calcium-exposed cases were averaged and are shown above as the normalized intensity versus 2θ. A *single asterisk* indicates significance when $p < 0.05$ or *double asterisks* when $p < 0.01$. *LF/NC* low fluoride and normal calcium, VHF/LC very high fluoride and low calcium

demonstrated an example of chronic fluoride overdose induced by excessive tea consumption [26]. The described deterioration of teeth due to chronic fluoride exposure was similarly identified in the present investigation. Specifically, the enamel, which is the outer component of the crown and the most radiodense structure on contact radiographs, was most significantly decreased in size as identified on the radiographs of the mandible (Fig. 3), while the tooth dentin showed only marginal changes with very high fluoride and low calcium exposure. The enamel is predominantly composed of hydroxyapatite, and its mineralization is highly sensitive to free fluoride ions. As the enamel is located on the outer surface of the tooth, it has a high susceptibility to abrasive effects. Since calcium is part of hydroxyapatite, a deficiency in calcium can potentially enhance the negative effects of chronic high fluoride exposure to teeth. In mineralized tissue, fluoride partially substitutes for the hydroxyl ion within the mineral lattice and results in larger crystal sizes, with a substantial effect on chemical stability [27]. Here, the EDX analyses indicated that the structure in the enamel and dentin had higher fluoride levels in the very high fluoride and low calcium-exposed sheep, as compared to the low fluoride control teeth (Fig. 1c). The effect was even more prominent in alveolar bone specimens, based on EDX and qBEI analyses, due to lifelong remodeling of bone tissue. These results are consistent with previously published data showing that very different lattice packing and fluoride sensitivities occur among the spongy bone, compact bone, teeth dentin and hard enamel [27]. The very high fluoride and low calcium exposure caused substantial negative effects on the analysed alveolar bone structures with significant cortical thinning and bone volume decrease (Fig. 4). High fluoride and low calcium exposure not only have negative effects on the alveolar bone but also have systemic skeletal effects. Our previous studies have shown that high fluoride and low calcium levels in drinking water are associated with low bone mass and reduced bone quality in long bones and spine in sheep [11]. This is in contrast to results from humans treated with a low- and high-dose sodium-fluoride regimen with sufficient calcium levels [28]. A comparison of the studies, however, is limited due to different study designs. In the human studies, analyses were not performed on the alveolar bone. Furthermore, we have analysed a lifelong exposure of very high fluoride water levels in combination with low calcium levels. The fluoride concentrations in our study were 13 times above the new U.S. HHS recommendation for a single level of 0.7 mg of fluoride per liter of water [25]. Such an experimental setup would not be realizable in human studies.

a

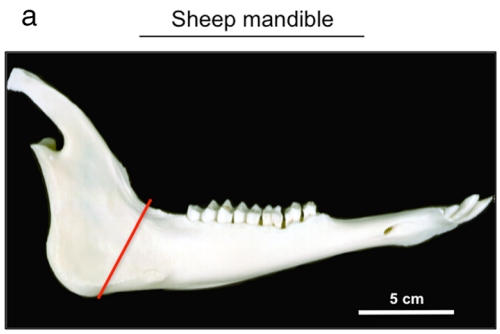


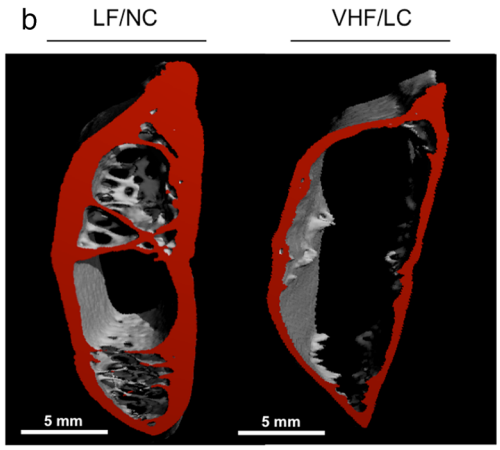

b

| | Incisor | | Premolar | | Molar | |
|---|---|---|---|---|---|---|
| Tooth length (mm) | *Crown* | *Root* | *Crown* | *Root* | *Crown* | *Root* |
| Low Fluoride/Normal Calcium | 10.9 ± 3.4 | 32.2 ± 2.1 | 3.2 ± 2.2 | 10.9 ± 3.4 | 8.5 ± 5.1 | 14.8 ± 3.8 |
| Very High Fluoride/Low Calcium | 2.8 ± 8.1* | 28.9 ± 4.8 | 0.5 ± 1.1* | 5.7 ± 5.5 | 1.7 ± 2.4* | 12.8 ± 0.5 |

**Fig. 3** Mandible and tooth deterioration. Macroscopic images, contact x-rays, Xtreme-CT scans and ground sections (**a**) of low fluoride and normal calcium demonstrate good tooth and alveolar ridge height. The influence of very high fluoride and low calcium is demonstrated in (**a**). Tooth lengths were measured on radiographic images and demonstrate a significant reduction, mainly seen as a crown height reduction in the very high fluoride and low calcium-exposed flock (**b**). A *single asterisk* indicates significance when $p < 0.05$ or *double asterisks* when $p < 0.01$. *LF/NC* low fluoride and normal calcium, *VHF/LC* very high fluoride and low calcium

In the XRD data, the intensity (whereupon peak intensities refers to how much the matter can be seen by comparing isotypic substances) of the very high fluoride/low calcium-exposed enamel was lower, indicating a change in mineral construction possibly through changes in the crystal structure, less crystalline hydroxyapatite in the enamel or just fewer mineral particles. In similar experiments, Wakamatsu and colleagues and Larsen and Jensen [29, 30] were also able to detect a decreased crystallinity of enamel apatite in the XRD data. Larsen and Jensen analysed the solubility of fluorhydroxyapatite as a function of the fluoride concentration in the apatitic lattice. Thereby, XRD showed that the length of the alpha-axis decreased with increasing fluoride concentration, while a broadening of the reflection indicated the presence of mixtures of various fluorhydroxyapatites [31]. Wakamatsu and colleagues studied the crystallinity of fluorosed enamel of rats, caused by the ingestion of fluoride containing water. Enamel was also evaluated with microbeam XRD analysis and by SDS-polyacrylamide-gel electrophoresis. There results suggested that in hypomineralized enamel caused by long-term administration of fluoride containing water, the degradation of amelogenin protein was disturbed, and consequently the crystallinity of enamel apatite decreased [31]. While our current data do not demonstrate a peak shift [32], the differences in the spectra support the idea that very high fluoride and low calcium intake has changed the mineral structure (Fig. 2). Very high fluoride and low calcium exposure in the tooth formation period inhibits proper formation of the enamel, leading to structural deficits in development and hypomineralization [21, 33]. The mineralization problem is particularly enhanced when there is a concurrent deficit in calcium uptake [31]. This combined effect of very high fluoride and low calcium on enamel was confirmed by the results of our study. The excessive systemic exposure to fluorides in combination with low calcium levels may be the main cause for the deterioration of the teeth, especially during the tooth formation phase of the Dorper sheep. However, if recommended levels of fluoride (which are much lower than those studied by us) in drinking water or other sources are maintained, the protective benefits (e.g. less caries decay) prevail [34, 35]. Kierdorf and colleagues described higher enamel fluoride concentrations and similarly disturbed enamel formation




c

| | LF/NC | VHF/LC |
|---|---|---|
| C.Th (mm) | 1.57 ± 0.34 | 0.80 ± 0.16** |
| Tb.N (1/mm) | 0.37 ± 0.13 | 0.27 ± 0.06 |
| Tb.Th (μm) | 215 ± 46 | 170 ± 21* |
| Tb.Sp (μm) | 2854 ± 1222 | 3848 ± 894 |
| BV/TV (%) | 7.6 ± 2.3 | 4.6 ± 1.4* |

**Fig. 4** Structural analysis of the mandible. **a** Macroscopic image of the mandible of a Dorper sheep; the *red line* indicates the cross-section analysed by HR-pQCT. **b** 3D cross-cut of the mandible showing cortical thickness and trabecular network reductions in the very high fluoride and low calcium-exposed sample. **c** Table of structural parameters detected by the HR-pQCT evaluation. A significant reduction was detected in cortical (*C.Th*) and trabecular thickness (*Tb.Th*) and in the bone volume (*BV/TV*) in the very high fluoride and low calcium flock in comparison with the low fluoride flock. A *single asterisk* indicates significance when $p < 0.05$ or *double asterisks* when $p < 0.01$. *C.Th* cortical thickness, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular spacing, *BV/TV* bone volume per tissue volume, *LF/NC* low fluoride and normal calcium, *VHF/LC* very high fluoride and low calcium

with isolated pits in wild boars, which were exposed to severe atmospheric fluoride deposition [33].

The effects of fluoride on enamel development have been studied in a wide range of animal species and tooth types. In most studies, rat incisors and molars were analysed. Other studies used incisors and molars of sheep pig molars, rabbit incisors and molars, hamster molars, mouse incisors and zebrafish teeth [36]. With the exception of zebrafish, which do not form true enamel, most species develop enamel in similar ways, although there are some differences between species and the type of tooth studied [36]. Rodent incisors have been used as a model system for several reasons, including their rapid and continuous eruption, allowing for the study of the effects of fluoride on amelogenesis even in adult animals. Other useful models are developing molar teeth of hamsters, with faster tooth development than rats, and with a cusp morphology more similar to that of human than of rat and mouse molars, as well as teeth of larger animal species, such as sheep and pigs [36]. The effects of systemic high fluoride exposure on bone and teeth could not only be observed in the abovementioned animal models but could also be observed in other domestic animals. Chronic fluoride toxicity in the form of dental and skeletal fluorosis was e.g. observed in cattle, buffaloes, sheep and goats from 21 villages of Banswara, Dungarpur and Udaipur districts of Southern Rajasthan (India) where the mean fluoride concentration in drinking water varied from 1.5 to 4.0 ppm [37].

Fluoride uptake in the enamel takes place during osteogenesis until tooth formation is completed. However, teeth are subject to increased attrition with a loss of surface area, and post-eruptive enamel fluoride uptake can be an aggravating factor [38, 39]. Diet can be an abrasive factor and augment structural loss, but the diet here did not differ significantly between the low fluoride/normal calcium and very high fluoride/low calcium group. However, lifelong exposure to highly fluoride and low calcium water can influence and worsen the quality and appearance of teeth (dental/enamel fluorosis) in the developmental years and even after tooth development is complete [40, 41]. These changes can be even more pronounced in bone due to its continuous remodeling [28]. This effect of fluoride on bone was partly demonstrated with decreased bone strength in three-point-bending tests of rat femurs after high fluoride intake and increased bone strength with low fluoride intake, but this was not simultaneously accompanied by an increase in bone density [42, 43].

The US Department of Health and Human Services (DHHS) recently finalized a recommendation for lowering the levels of water fluoridation programs to 0.7 mg/l for the whole USA due to potential health problems of severe dental fluorosis associated with chronic higher levels of fluoride intake [7, 25]. The data presented here confirm these concerns and are of particular importance for people living in regions with natural high fluoride content due to geologic/pyroclastic activity in the past [7].

Although there were several study limitations, the results are noteworthy and of special importance. First, we focused on characterizing the dentoalveolar status of 6- to 7-year-old sheep and did not have the chance to analyse different ages of sheep. Second, alveolar bone deterioration can also be (partially) influenced by crown attrition due to tooth decay or enamel hypoplasia, as altered mechanical properties for mastication lead to load changes and negative alveolar bone adaption [44]. Information on the hardness or mineral density of the enamel would be important to explain the severe attrition. Different diets, as an attributed confounder, have to be

taken into account, but that is unlikely in our experimental setup since the foods for both Dorper groups were alike due to identical environmental situations. Furthermore, data from different bone samples (spine, femur) have identified direct negative effects on the trabecular and cortical bone due to lifelong very high fluoride exposure [11]. Third, as the sheep in the Kalahari Desert were exposed to very fluoride-rich groundwater poor in calcium, the observed dental phenotype can be explained by the combination of both very high fluoride and low calcium exposure [45]. Comparative in vivo analyses for this hypothesis cannot be addressed in freely farming sheep, and it would require an experimental setting with sheep housed in metabolic cages allowing for control of nutritional input and balance [46].

In summary, the current study demonstrates the effects of chronic very high fluoride and low calcium exposure and identifies fluoride 13 times above recommended levels as potentially dangerous [25]. The analyses show that a combined chronic environmental very high fluoride and low calcium intake leads to a severe dental fluorosis, which presents as phenotypical and structural changes, particularly in tooth enamel, as described by others before. Furthermore, it induces significant structural attrition of teeth and an astoundingly significant deterioration to the dentoalveolar apparatus with reduced mandibular bone quality. In the presence of high fluoride and low calcium-associated dental changes, a potential paradox alveolar bone loss needs to be clinically considered regardless whether other signs of skeletal fluorosis.

**Acknowledgments** We would like to thank Marion Dietzmann and Mona Neven for their expert help in histological processing of the bone samples. We are thankful for the advice of Björn Busse on the work with the electron microscope. Additionally, we would like to thank Dr. Ulrich Borchers and Dr. Achim Rübel for their help with the groundwater analyses at the IWW Water Research Institute, as well as Dr. Helmut Dietrich Köster and Dr. Peter Heitland at Medizinisches Labor Bremen for the analysis of blood and urine. Finally, we thank the farmers in the Kalahari area for their unrestricted support and friendly cooperation.

**Compliance with ethical standards**

**Ethical approval** All applicable national and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. This study was carried out according to existing rules and regulations of the University Medical Center Hamburg-Eppendorf and is in line with the "Hamburg Hospital Law (HmbKHG) April 17th, 1991: Patient Security §12" and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Conflict of Interest** The authors declare that they have no competing interests.

**Funding** No funding was applicable.

**Informed consent** Informed consent was obtained from all subjects and/or their parents.

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