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Defective enamel ultrastructure in diabetic rodents

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Summary. We investigated six different types of diabetic rodents. Four expressed a genetic obesity resulting in diabetes. One developed diabetes induced by a diet-dependent obesity, and one with genetic diabetes received anti-diabetic medication. The tooth samples were examined under a scanning electron microscope and with an energy dispersive microanalysis (EDX). The electron micrographs showed severe, varying degrees of damage within the six different diabetic animal types, such as irregular crystallite deposition and prism perforations in genetically obese animals compared to less-disordered prism structures in diet-dependent obesity. Anti-diabetic medication resulted in normal enamel ultrastructure. The EDX analysis revealed a reduction in the amount of calcium and phosphorus in all regions affected by diabetes. Based on these animal studies, we suggest that both juvenile diabetes type I (in infants) and adult diabetes type II (in pregnant mothers, affecting the developing foetus) may affect the normal development of teeth in humans.

Introduction

Very few studies have so far been able to clearly demonstrate the effects of diabetes mellitus on developing dental hard tissues and bone. Some investigations focused on bone growth and enamel mineralization under diabetic influence in rat models [1–3]. Other studies used *in vitro* assays to prove glucose-induced inhibition of bone mineralization [4]. Another approach comprised *in vivo* investigations on the influence of mainly maternal diabetes mellitus, but also of other sometimes correlated diseases on primary teeth. These studies contained macroscopic and microscopic observations by light microscopy, microradiography and also microhardness tests [5–8].

However, no scanning electron microscopy (SEM) examinations have so far been performed to investigate ultrastructural alterations in mineralized tissues. The above-mentioned papers draw rather contradictory conclusions, and therefore, further investigations needed to be carried out to clear up the question of how diabetes mellitus may influence enamel formation and ultrastructure.

In this study, the influence of diabetes mellitus has been studied by means of SEM to provide a clear understanding of possible enamel destruction. We investigated the defects in several animal models suffering from diabetes mellitus and obesity. In addition, we wanted to see if specific differences between enamel ultrastructure caused by diabetes mellitus type I and type II do exist.

Materials and methods

Test and control animals

B6.Cg-m+/+*Lepr*^{db} mice, B6.V-*Lep*^{ob} mice, ZDF/Gmi-*fa* rats, ZUCKER-*fa*/*fa* rats and 12-week fatty diet rats all suffer from genetically or dietary induced obesity leading to diabetes mellitus. In addition to these test animals, Rosiglitazone™ was

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administered to a part of the B6.V-*Lep*^{ob} strain to investigate possible anti-diabetic effects on the enamel ultrastructure. Mice and rats were obtained from Metabolic Preclinical Research, Vascular and Metabolic Diseases, Pharmaceuticals Division, Hoffmann-La Roche Ltd, Basel, Switzerland, where they were reared.

We chose BL/6 mice and Sprague Dawley rats as control animals. Both strains were kept for some time at the Institute of Anatomy of the University of Basel, before they were investigated. During this period, they were fed standard rodent diet.

Calvarial and dental maceration

After decapitation, the heads of the animals were boiled in distilled water for 30 min. Sodium peroxide was added to the water for another 10 min. Calvarial bone was separated from fur and muscle tissue. The sculls were dried in an oven at 60 °C for 30 min. Maxillary and mandibular teeth were separated from the alveoles. Longitudinal abrasion was performed with a laboratory micromotor handpiece (Micro-M3ASF, Badeco, Geneva, Switzerland) and rotating discs (Sof-Lex™, 3E Dental Products, St Paul, MN, USA) at 8000 r.p.m. The ground teeth were polished with ultrafine discs. The teeth were subsequently washed and cleaned of all polishing dust in an ultrasonic cleaner (Bransonic 2200 E4, Branson Cleaning Equipment Company, Shelton, CT, USA) for 5 min. They were further cauterized with 2 N hydrochloric acid (HCl, E. Merck AG, Dietikon, Switzerland), according to the procedure used by Martin [9], for 6 s to set off the enamel prisms and to remove the smear layer.

Scanning electron microscope preparation and examination methods

All tooth samples were mounted on specimen stubs and sputtered for 120 s at 20 mA, 2·4 kV and a vacuum value of 7×10^{-2} with a 12 nm thick gold layer (Polaron SC515 SEM Coating System, Fisons Instruments, Ipswich, UK).

The specimens were then examined with a scanning electron microscope (Philips XL 20, Philips, Eindhoven, the Netherlands) using either secondary electron mode (SE) or with back scatter electron mode (BSE). The magnification ranged between $\times 20$ and $\times 3000$. The microscope was operated at an accelerating voltage of 10·0 kV.

Energy dispersive X-ray spectrometry (EDX) was carried out in combination with SEM (EDAX™ DX-4I, EDAX™ International Inc., Mahwah, NJ, USA). Within all specimens investigated with EDX, 120 life seconds have been counted.

Results

Enamel ultrastructure of diabetically impaired rodents

In general, all enamel samples from rodents influenced by diabetes mellitus showed a clearly modified ultrastructure as compared to those taken from healthy control animals (Fig. 1). Interestingly, we found distinct enamel alterations in each of the different animal types, such as: prism perforations in B6.Cg-m+/+*Lepr*^{db} mice (Fig. 2); prism structures altered from ovoid to sharp tips in B6.V-*Lep*^{ob} mice; altered periodicity of the bands of Hunter and Schreger from uniserial to pauciserial in ZDF/Gmi-*fa* rats (Fig. 3); two-dimensional defects extending over the surface in ZUCKER-*fa*/*fa* rats; and loosely packed prisms in 12-week fatty diet rats.

The diabetic mouse strain receiving Rosiglitazone™ showed surprising features. In the inner enamel layer, the SEM equivalents of the bands of Hunter and Schreger had absolutely normal proportions. These bands were uniserial and had an even more

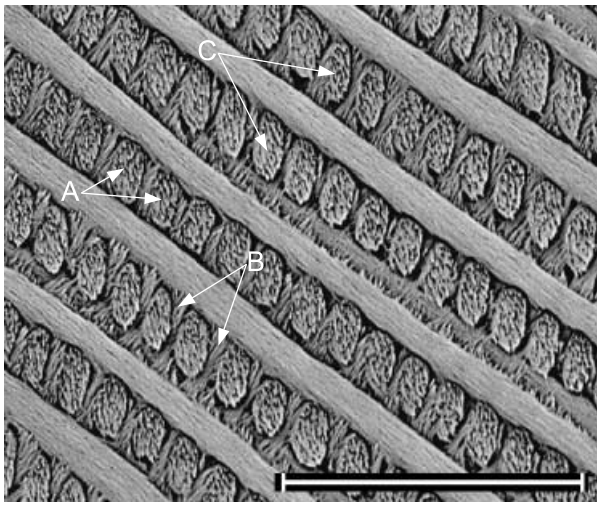

Fig. 1. Scanning electron microscopy (SEM) image of the apical enamel region of a mandibular tooth from a Sprague Dawley control rat. (A) Perfect arrangement of enamel prisms surrounded by interprismatic enamel. (B) SEM equivalents of the bands of Hunter and Schreger in a regular course (C) (bar = 20 μm).

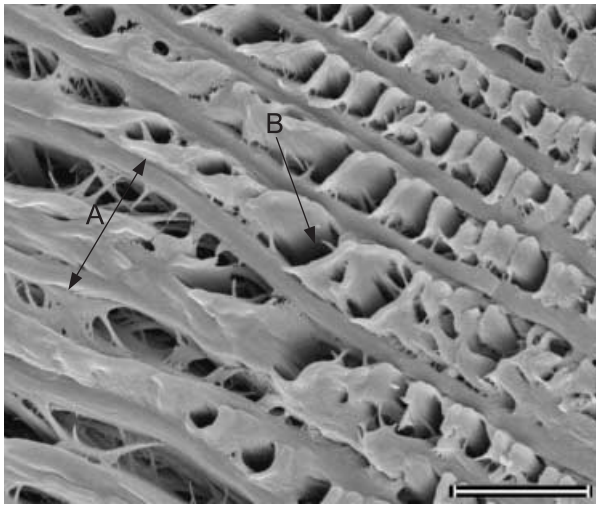

Fig. 2. Scanning electron microscopy (SEM) image of diabetically impaired enamel from a maxillary tooth of a B6.Cg-m+/+*Lepr*^{db} mouse. (A) The SEM equivalents of the uniserial bands of Hunter and Schreger appear thicker than normal, and (B) the prisms seem perforated (bar = 10 μm).

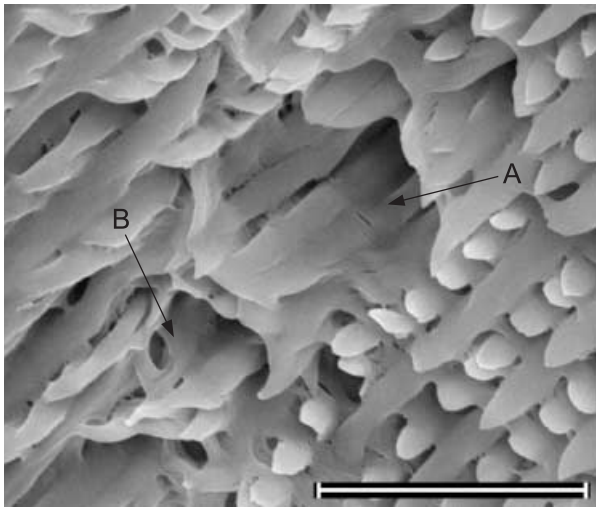

Fig. 3. Scanning electron microscopy (SEM) image of damaged enamel from a mandibular tooth of a ZDF/Gmi-*fa* rat. (A) The defects are not only restricted to the prisms themselves, but also spread over the prism borders. (B) The structural characteristics of the prisms and interprismatic enamel are hardly detectable (bar = 20 μm).

regular structure and surface than those of the untreated control animals. The prisms of this mouse strain's teeth showed not only the normal ovoid structure, but the crystallites of these rods seemed to be even more tightly packed than those of control teeth (Fig. 4).

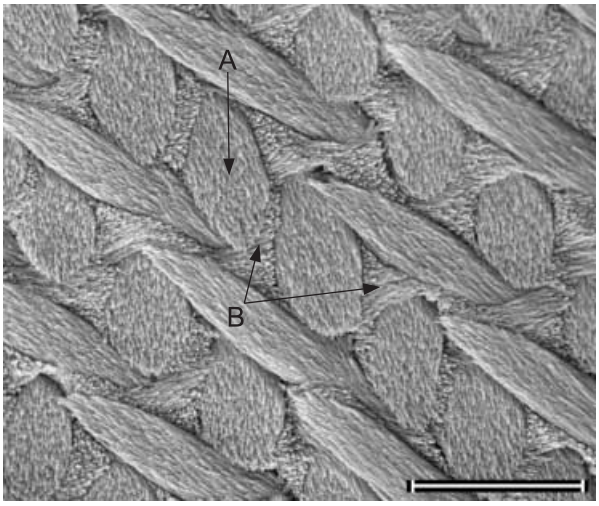

Fig. 4. Representative scanning electron microscopy image of an apical enamel region from a maxillary tooth obtained from a B6.V-*Lep*^{ob} mouse receiving Rosiglitazone™ as anti-diabetic medication. (A) The prism crystallites seem to be more tightly packed compared to non-diabetic control specimens, and (B) the interprismatic enamel lies smoothly around the rods (bar = 5 μm).

EDAX™ observations

In all investigated specimens, a characteristic spectrogram of calcium and phosphorus was detected. The largest amounts of both elements were detected in the coronal and apical regions of control teeth. The peaks for phosphorus were always lower than those for calcium. On the other hand, mice always showed lower peaks for both elements than rats.

The apical enamel regions of the diabetic rodents, i.e. the enamel regions formed under the influence of diabetes, showed a massive reduction in the amount of calcium and phosphorus compared to the coronal parts of teeth formed before the onset of diabetes. The influence of diabetes on phosphorus seemed to be much more dramatic since phosphorus peaks were much more decreased than those of calcium. The biggest decrease of phosphorus peaks, and therefore the biggest increase of the calcium to phosphorus ratio was found in the ZDF/Gmi-*fa* samples, which also corresponds to the most dramatic ultrastructural changes found in our study. In all other specimens, there was also a remarkable decrease in phosphorus, but this could not be correlated to the degree of ultrastructural defects, however.

Rosiglitazone™-treated animals likewise showed strongly decreased calcium and phosphorus peaks, which could not be correlated to any features in the pattern of enamel ultrastructure.

Discussion

Ultrastructural evidence of diabetically impaired enamel

The strongest ultrastructural destructions were observed in ZDF/Gmi-*fa* rats, where large areas including whole prisms were destroyed. B6.Cg-m+/+*Lepr^{db}* mice, ZUCKER-*fa*/*fa* rats, B6.V-*Lep^{ob}* mice and 12-week fatty diet rats showed smaller ultrastructural destructions. The extent of these destructions is decreasing in the order mentioned above, with the least in the 12-week fatty diet rats. From the micrographs shown in this study, it is evident that all animals affected by a genetically determined diabetes show markedly stronger defects than the animals developing diabetes after a fatty diet. From these findings, we have drawn the conclusion that, in mammals, a genetically induced diabetes like juvenile type I diabetes may lead to much more destruction than diabetes type II, which is normally acquired during middle-age, and could affect developing foetuses in the mother.

Investigations carried out by other authors have concentrated on the effects of diabetes type II of the mother on developing foetuses and not on the direct influence of juvenile diabetes type I on the formation of teeth. Thus, Grahnen and Edlund [10] found in their study that enamel hypoplasia was more common in the children of diabetic mothers than in the controls. Furthermore, they concluded that both prenatal and postnatal enamel hypoplasia is relatively common in children of diabetic mothers.

The work of El-Bialy *et al.* [11] unequivocally supports our suggestions concerning stronger defects in juvenile diabetes mellitus. They found decreased skeletal maturation and decreased cephalometric measurements in diabetic patients with juvenile diabetes. Their results may possibly be transferable to dental enamel, since bone tissue and enamel show similarities in their function as mineralized tissues. Likewise, the recent study from Giglio and Lama [1] proved that diabetic rats showed significantly reduced growth in most of the mandible skeletal units. Mandibular growth as a whole was also significantly lower and disharmonious in diabetic animals compared to controls. Interestingly, the investigation by Adler *et al*. [12] showed a clear relationship between the age of the child and the influence on dental development. Diabetic children grouped according to their age at dental examination demonstrated a gradual retardation of dental development with advancing age. A progressive retardation of dental development occurred with increased duration of the disease. The recent investigations of Gunczler *et al*. [13] also referred to decreased bone mineral density and bone formation markers shortly after diagnosis of clinical type I diabetes mellitus.

A very interesting observation was the enormous effect of the anti-diabetic treatment on dental enamel, since this medication is known to counteract the secondary effects of diabetes such as microangiopathy. As a result of the Rosiglitazone™ application, the newly formed prisms and the interprismatic enamel were arranged in a perfect order. Thus, anti-diabetic medication actually counteracts the adverse effects of diabetes on enamel formation. Our opinion is supported by the clinical study by Grahnen *et al*. [14], and the histologic and microradiographic study by Noren *et al*. [15]. These investigators could prove that enamel showed fewer disturbances in the children of mothers with diabetes type II who had been treated with anti-diabetic medication during pregnancy.

In all diabetic animal types with defective enamel regions, all prisms involved showed marked alterations, which obviously resulted from the destruction of the three-dimensional crystallite arrangement. We believe that the following three hypotheses may be of importance for altered crystallite arrangement within prisms. For prism formation and arrangement:
1 minerals and elements such as calcium and phosphorus are absent, or
2 secondary effectors such as proteins, which control the exact arrangement of the prisms, are absent. In the latter case, the function of the ameloblasts would also be remarkably disturbed.
3 Another possibility is that hyperglycemia could directly affect prism formation in a negative way. In this case, the increased blood glucose concentration would be directly responsible for the irregular prism structure by influencing the secretory ameloblasts.

The work of Noren [16] supports the first hypothesis, since he found in children of diabetic mothers a significant increase in the usually occurring hypoplasia in connection with the neonatal line, as well as a general impairment of calcium and phosphorus homeostasis. Additionally, Noren found a decreased calcium level in mothers with diabetes mellitus type II. It is, therefore, reasonable to assume that the decrease in the mothers' plasma calcium is responsible for a structural defect in the enamel of their children. This hypothesis is strongly contested by

the study of Verhaeghe *et al*. [3], who claimed that there is no mineralization defect caused by calcium deficiency in foetuses of diabetic rats, but a delay in bone maturation, as indicated by a lower number of ossification centres. Calcium and phosphor deposition in bone, however, must not be the same as it is in enamel formation.

The work of Sasaki *et al*. [17] clearly contradicts the second hypothesis. They claimed that, although a study by Karim [18] had suggested that experimentally induced diabetes inhibits enamel protein secretion by secretory ameloblasts, the structure of these cells did not appear markedly abnormal. In addition, they cited Warshawsky [19], who demonstrated that the secretory ameloblasts were lacking insulin receptors.

A recently published study by Balint *et al*. [4] supports the third hypothesis. This study is the first to demonstrate that elevated glucose concentration inhibits osteoblastic calcium deposition and bone maturation. They demonstrated that a glucose concentration similar to those observed in patients with poorly controlled diabetes causes significant inhibition of osteoblastic calcium deposition.

X-ray spectrometry

Our findings support the hypothesis that a decrease of calcium and phosphorus levels may be directly responsible for the enamel defects. We suggest that diabetes mellitus directly causes this decrease of calcium and phosphorus levels, which causes the enamel defects in turn.

In the study of Sato *et al*. [2], the alterations in the mineralization pattern of enamel were examined by microradiography and electron probe microanalysis. They suggested that enamel malformation is caused by decreased cellular activity with regard to the regulation of calcium transport under toxic regimens. The effects of these toxic regimens can also be compared to the detrimental influence of an untreated diabetes mellitus. Shyng *et al*. [20] examined the effect of Streptozotocin-induced experimental diabetes mellitus on calvarial defect healing and bone turnover in the rat. They found that cancellous bone volume and bone formation in the femur were greatly reduced in the diabetic model. Assuming that osteoid and the organic matrix of enamel are somewhat comparable as far as the deposition of calcium and phosphorus is concerned, we can again come to the conclusion that less calcium is integrated in both the bone and enamel of diabetic animals. Lassila and Virtanen [21] investigated the impact of Streptozotocin-induced diabetes on rat blood and on alveolar bone affected by occlusal stress. They found a significant increase in the serum glucose and total calcium levels, possibly deriving from a deficiency in the kidneys and the liver. In the alveolar bone of the diabetic rats, a clearly retarded bone matrix and new bone formation was found. Needleman *et al*. [22] examined antecedents and correlates of hypoplastic enamel defects in primary incisors. They found that neonatal hypocalcemia is common in new-borns and is more prevalent in those who are premature than in those born at term, and that these low calcium levels can directly affect the enamel formation and lead to hypoplastic enamel defects.

Our assumption that a decreased calcium level in the blood or a decreased calcium incorporation into the enamel caused by decreased cellular activity of the ameloblasts may lead to the above-mentioned defects in diabetes mellitus is further consolidated by investigations considering the state of enamel under the influence of other diseases. In their measurements of the enamel mineral composition of normal and cystic fibrosis transgenic mice, Wright *et al*. [7,8] found that mice with cystic fibrosis had markedly hypomineralized enamel. A paper by Bhat and Nelson [23] reviewed developmental enamel defects in the primary teeth of children with cerebral palsy, learning difficulties and hearing defects. This review indicated that developmental hypoplastic enamel defects in the primary teeth occur with greater frequency in children with cerebral palsy, learning difficulties or sensory-neural deficits than in comparison groups.

Conclusion

From the findings presented in this study, we strongly recommend new dental management considerations for the child patient with impaired enamel caused by diabetes mellitus. It is a fact that the world-wide prevalence of diabetes mellitus is increasing steadily [24]. The increasing longevity of the global population and more effective diagnostic protocols mean that the dental practitioner will be treating an increasing number of patients with this disease. Simultaneously, the number of patients who suffer from diabetes mellitus during the developmental stages of enamel formation will increase. Considering the dramatic defects in enamel caused by diabetes mellitus, we should be aware that young patients

with diabetes mellitus may suffer from different degrees of damaged enamel ultrastructure and mineral composition, depending on the extent and course of the disease, and that, in turn, these damaged regions may cause vulnerability to caries, erosion, abrasion or even fracture.

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Résumé. Nous avons étudié six différents types de rongeurs diabétiques. Quatre d'entre eux exprimaient une obésité génétique résultant en un diabète, un avait développé un diabète consécutif à une obésité liée au régime alimentaire, et un avec un diabète génétique recevait un traitement antidiabétique. Les échantillons dentaires ont été étudiés sous microscope électronique et par microanalyse dispersive d'énergie (EDX). Les images de microscopie électronique ont montré des dommages sévères à des degrés variés chez chacun des six types d'animaux diabétiques, à type de dépôts de cristaux irréguliers et perforations des prismes chez les animaux obèses génétiquement en comparaison aux structures prismatiques moins désordonnées chez ceux dont l'obésité dépendait du régime. Le traitement antidiabétique était associé à une ultra-structure normale de l'émail. L'analyse EDX a révélé une réduction de la quantité de calcium et de phosphore dans toutes les régions affectées par le diabète. A partir de ces études chez l'animal, nous suggérons que, chez l'humain, à la fois le diabète juvénile de type I (jeune enfant) et le diabète adulte de type II (femmes enceintes, affectant le fœtus) peuvent affecter le développement normal des dents.

Zussamenfassung. Wir untersuchten sechs verschiedene Arten von Nagern mit Diabetes, bei vier davon als Folge eines genetisch bedingten Übergewichtes, einmal als Folge eines ernährungsinduzierten Übergewichtes und einmal mit genetisch bedingtem Diabetes unter antidiabetischer Therapie. Zahnproben wurden mittels Rasterelektronenmikroskop und energiedispersiver Röntgenmikroanalyse untersucht. Rasterelektronenmikroskopisch zeigten sich schwere, unterschiedlich stark ausgeprägte Schäden bei den verschiedenen Tiergruppen, bei den genetisch bedingten Diabetesformen waren die Kristallitenablagerungen irregulär, weniger stark war dies im Vergleich dazu bei dem rein ernährungsbedingten Diabetes. Antidiabetische Behandlung ergab normale Schmelzmorphologie. Die Röntgenmikroanalyse zeigte eine Reduktion von Calcium und Phosphor in allen von Diabetes betroffenen Regionen. Aufgrund dieser Tierversuche vermuten wir einen Einfluss auf die Zahnentwicklung auch beim Menschen, und zwar sowohl beim juvenilen (Typ I) Diabetes als auch beim Typ II Diabetes (bei betroffenen Schwangeren mit Auswirkungen beim Fetus).

Resumen. Investigamos seis tipos diferentes de roedores diabéticos, cuatro de los cuales expresaban una obesidad genética que producía diabetes, uno desarrolló diabetes inducido por una dieta que producía obesidad y otro con diabetes genética recibió medicación antidiabética.

Las muestras dentarias se examinaron con un microscopio electrónico de barrido (SEM) y con un microanálisis de dispersión de energía (DEX). Las micrografías de electrones mostraron severidad, varios grados de daño en los seis tipos diferentes de animales diabéticos, tales como deposición irregular de cristales y perforaciones de los prismas en animales genéticamente obesos comparados con estructuras prismáticas menos desordenadas en la dieta productora de obesidad.

La medicación antidiabética produjo una ultraestructura del esmalte normal. El análisis DEX reveló una reducción en la cantidad de calcio y fósforo en todas las regiones afectadas por diabetes. Basados en estos estudios de animales, sugerimos que en los humanos tanto la diabetes juvenil tipo I (en lactantes) y la diabetes tipo II (en madres embarazadas, que afecta al desarrollo del feto) pueden alterar al desarrollo normal de los dientes.

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