Abstract

Complications of diabetes mellitus that affect the different body organs represent an increasing threat because of the increasing prevalence of this condition worldwide. As rodent incisors are continuously growing teeth that include all stages of amelogenesis, they could be used as a model for the effect of diabetes on tooth development. In the present experimental study, we analyzed the ameloblasts and the enamel surface of the mandibular incisors of rats with Streptozotocin-induced diabetes. Materials and Methods: Groups consisted of 30 streptozotocin induced diabetic albino rat and 15 controls in which sterile saline solution was administered instead of streptozotocin. The enamel surface of the rats’ mandibular incisor was analyzed by the scanning electron microscope (SEM). The ameloblasts were examined histologically by the light microscope and immunohistochemically by Cytokeratin 14 (K14) detection. Results: By SEM the enamel surface showed variable degrees of hypoplasia ranging from mild to severe hypoplasia. By the light microscopy, the ameloblasts demonstrated shrinkage, cytoplasmic vacuolization and smaller nuclei than those of the control group. There was also widening of the intercellular spaces. In addition, the immunohistochemical staining pattern for K14 was altered in ameloblasts of the diabetic rats. Conclusions: Diabetes may have a detrimental influence on the function of ameloblasts in laying down enamel causing morphological changes in enamel and suggesting a reduction in secretion and mineralization.

Introduction

Diabetes mellitus is a heterogeneous clinical syndrome characterized by endocrine and metabolic changes that affect homeostasis. The main endocrine problem is partial or total insulin deficiency, leading to profound changes in metabolism of carbohydrates, proteins and lipids. From the frequent association of diabetes mellitus and pregnancy, a clinical class termed gestational diabetes mellitus has emerged, which includes patients who develop or are first diagnosed during pregnancy with diabetes mellitus or glucose intolerance. Birth defects associated with maternal diabetes mellitus in experimental animals have long been reported in the literature [1]. Diabetes may be
experimentally produced surgically by pancreatectomy, or chemically by administration of streptozotocin or alloxan \cite{2,3}.

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 \cite{4}. Other studies used in vitro assays to prove glucose-induced inhibition of bone mineralization \cite{5}. Another approach comprised in vivo investigations on the influence of mainly maternal diabetes mellitus, but also of other sometimes correlated diseases on primary teeth \cite{6}.

The formation and mineralization of enamel is controlled by epithelial cells of the enamel organ which undergo marked, and in some cases repetitive, alterations in cellular morphology as part of the developmental process. The most dramatic changes are seen in ameloblasts which reverse their secretory polarity during differentiation to allow for extracellular release of large amounts of proteins from plasma membrane surfaces that were originally the embryonic bases of the cells \cite{7}.

Continuously growing teeth are useful in the study of differentiation of dental progenitor cells. In rat lower incisors, ameloblasts originate from the dental epithelial adult stem cell compartment referred to as the ‘apical bud’ \cite{8,9}. Since the incisors of rodents present continuous development and a single section can show the various stages of the life cycle of ameloblasts as well as the phases of secretion, calcification and maturation of the enamel matrix \cite{10}, it is of fundamental importance that the analyses be conducted on the same region of the enamel organ. Thus, the morphological and morphometric analyses were limited to the final stage of secretion of the enamel organ since in this phase the enamel matrix has developed to its full thickness with a sufficient amount of preserved organic matter, which serves for histological determination of the structural organization of this tissue. The continuously growing incisor of the rodent is subdivided into a crown analog half on the labial side, with a cervical loop containing a large core of stellate reticulum, and its progeny gives rise to enamel producing. The lingual side is known as the root analog and gives rise to epithelial rests of Malassez \cite{11}.

Diabetes may have a detrimental influence on the function of ameloblasts in laying down enamel. Enamel hypoplasia in primary teeth of children born to diabetic mothers has been reported \cite{12}. Also Atar et al \cite{13} reported that diabetic rodents exhibit more wear in their teeth, random regions of hypomineralization and some areas of hypoplasia in the mandibular incisors.

Keratins are heteropolymeric proteins which form the intermediate filament cytoskeleton in epithelial cells. Cytokeratin (CK) is a filament which plays a central role in epithelial tissue and, like the polypeptides of intermediate filaments in general, shows a high degree of tissue specificity \cite{14}. Cytokeratin was used as a marker for epithelial cells \cite{8} as it is expressed only in the dental epithelium, and not in the mesenchyme of the tooth germ or any of the surrounding tissues in the mandible \cite{15}.

Domingues et al \cite{14} studied the distribution of individual CK polypeptides in the human enamel organ at bell stage and in remnants of the dental lamina. Their immunohistochemical study showed that epithelial cells stained for CKs 7, 13, 14 and 19 with slight changes in their pattern during the differentiation phase of odontogenesis. There was negative staining for all other CK polypeptides tested (CKs 8, 10, 16, 17 and 18). Most of the CKs in the enamel organ epithelia did not show differences related to the stage-specific state of differentiation, except for CKs 14 and 19 at the inner enamel epithelium. A strong label for CK 14 was present at the inner dental epithelium at early bell stage, and this was substituted by CK 19 at the late bell stage when the ameloblasts were fully differentiated.
Since 1991, mutations in several keratin genes have been found to cause a variety of human diseases affecting the epidermis and other epithelial structures. In some skin diseases for which the underlying genetic lesion was found, there were mutations in K5 and K14 genes rendering basal epidermal keratinocytes less resilient to trauma, resulting in skin fragility [16].

In the present study, we proposed to determine and characterize morphologically, histologically and immunohistochemically the ameloblast and enamel disorders in the mandibular incisor teeth of Streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Animals

Forty five male albino rats were used in this study. The rats were recorded (through one month period) at their birth day in the animal house of “The Medical Research Center” Faculty of Medicine, Ain shams university. After one month period (the lactation period), the animals were collected and housed in separated dated cages. 5 rats each. They were fed standardized diet and tap water ad libitum. The animals were left till 5-6 months age. Temperature and humidity conditions were controlled as possible on housing the animals during the experiment period. The animals were divided into two groups: Group I: control group which consists of fifteen rats age -matching those of the diabetic. The control rats received a single intraperitoneal injection with sterile saline instead of streptozotocin. Group II: diabetic group which consists of thirty rats received a single intraperitoneal injection of streptozotocin.

Induction of Diabetes

Group II animals were left over night fasting. Diabetes was induced by a single intraperitoneal injection of streptozotocin (58mg/kg body wt.) in citrated buffer. Animals were then left diabetic for eight weeks. Blood glucose level of the diabetic rats was monitored by periodic examination of blood samples, every two weeks. The blood sugar level was measured using Glucochek SC (Glucko test 20-800R). Animals were not considered diabetic unless they demonstrated serum glucose level greater than 250 mg/dl (normal serum glucose level was 65/110 mg/dl) according to Trinder [17] and Anderson [18].

Preparation of Specimens

The animals were killed by decapitation at the end of the experimental period. The mandible from each rat was removed. For the scanning electron microscopic (SEM) examination the right half of the mandibles was sectioned in the anterior segment to free the crowns of the incisor, taking care not to damage it to avoid artifacts. Before SEM preparation, the teeth were cleaned by spraying with water for 2 to 3 min. They were then brushed with rubber without any abrasive substance. They were left to dry on a filter paper, after which they were sputter-coated with gold for three 3-min cycles (SPI- module sputter gold coater). Analysis was performed under a JEOL (JSL-5500 LV) scanning electron microscope in the Regional Center of Mycology and Biotechnology (RCMB) at Al-Azhar University.

For the light microscopic preparation, the left side of the mandibles was dissected in the area of the continuously growing lower incisors just anterior to the first molar so that the incisor will be cut horizontally to be sure that all ameloblasts in the section are in the same stage of their life cycle. Specimens were fixed in 10% buffered formaldehyde for 24 hours [the formaldehyde was buffered in pH 7.2 phosphate buffer saline (PBS)]. The specimens were carefully decalcified using a solution containing 8% EDTA buffered in pH 7.2 PBS for 21-30 days at 4°C [19]. After decalcification, the specimens were processed & sectioned (5μm thickness) for the subsequent histological and immunohistochemical staining procedures. The histological examination was done by staining with H & E and investigated under light microscope.
Immunohistochemical staining

DAKO LSAB + kit, peroxidase, universal, k 0679 was used. In the first step, 3% hydrogen peroxide was applied to cover the specimens for five minutes. Then specimens were rinsed with the wash solution which consists of phosphate buffer saline pH 7.4. Then the sections were immunolabelled using the ready to use primary antibody anti-CK14 monoclonal antibody (Sigma-Aldrich, Inc). The specimens covered with primary antibody were incubated at 4°C overnight and then rinsed with the wash solution. After that the sections were covered by the link and incubated for 30 minutes. The link consists of biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate buffer saline (PBS) containing carrier protein and 15mm sodium azide. Then the specimens were rinsed with the wash solution. Streptavidin conjugated to horse-radish peroxidase in PBS containing carrier protein and an antimicrobial agent was applied to the specimens and incubated for 30 minutes. Then specimens were rinsed with the wash solution. The specimens were covered with Hematoxylin counter stain and incubated for two to three minutes and then rinsed with distilled water, dehydrated in graded alcohol and cleared in xylene. Finally the specimens were mounted in canada balsam and examined under the light microscope.

RESULTS

Histological results

For the control group figure 1 demonstrates the normal histological picture of ameloblasts in the mandibular rat incisor’s labial side. Ameloblasts are tall columner, well arranged. They are attached to each other, and have elliptical large proximally located nuclei (Fig.3). The intercellular spaces between ameloblasts in the control group are narrow (Fig.5).

In the diabetic rats the ameloblasts showed marked changes. They showed shrinkage, some cytoplasmic vacuolization and smaller nuclei than those of the control group (Fig.2 & Fig.4). Some ameloblasts lost their proximal junctions to the adjacent cells correspondingly there was widening of the intercellular spaces (Fig.6). In few areas the ameloblastic layer demonstrated disruption and some ameloblasts ruptured with remnants of nuclei (Fig.7).

Examination of the enamel rods pattern of the control group revealed a basket weave lattice arrangement (Fig.8 A&B). On the other hand, the diabetic rats showed different rods arrangement (Fig.9).
Immunohistochemical results

Figure 10 demonstrated ameloblasts from a control rat, immunolabelled for cytokeratin 14 (K14). All of them are intensely stained (Fig.10). On the other hand ameloblasts from the diabetic group showed a different pattern of staining. In some of the diabetic samples ameloblasts showed an overall decrease in the staining intensity for K14 (Fig.11). Some others demonstrated a faint staining for K14 in the whole cytoplasm except the distal end which showed a moderate staining (Fig.12).
Fig. (5) Ameloblasts from the control group are crowded with large nuclei and narrow intercellular spaces. (H&E x1000)

Fig. (6) Ameloblasts from the diabetic group are shrunken, with small dark nuclei (arrow) and wide intercellular spaces (arrowhead). (H&E x1000)

Fig. (7) Some ameloblasts from the diabetic group are ruptured (arrow) with remnants of nuclei (arrow head). (H&E x1000)

Fig. (8) A light microscopic picture of enamel rods from the control group showing the basket-weave pattern. (H&E, Ax400; Bx1000)

Fig. (9) A light microscopic picture of enamel rods from the diabetic group showing a different pattern from the basket-weave pattern of those from the control group. (H&E x1000)
Enamel Morphological Changes

The teeth were classified by SEM as normal or with defects that ranged from mild to severe. Normal enamel in the control rats was characterized by a homogeneous, regular, smooth surface with small and regular round depressions of varying depth, corresponding to Tomes’ processes of ameloblasts (Figure 13A, B). Mild defects were characterized by the presence of occasional irregular and superficial depressions with a rough appearance to the affected areas (Figure 14). Lesions of mild defects were more widespread and intense. Lesions of moderate defects were superficial with variable size and form. Rod ends appeared within those defects (Fig.15 A, B). In some of the moderate defects, small pores and cracks were present (Fig.15 B). Severe hypoplasia was characterized by intense, multiple longitudinal defects with variable depth in which a deformed basket-weave pattern could be seen (Fig. 16). Using SEM, only 1(6.7%) of the 15 control teeth exhibited mild hypoplasia (Fig.13 B). In the diabetic group, 2 (6.7%) teeth were normal whereas mild hypoplasia was seen in 14 (46.7%), moderate in 9 (30%) and severe in 5 (16.7%).
FIG. (13) Scanning electron microscopy of the enamel surface of a rat’s mandibular incisor from the control group showing a homogenous relatively smooth surface with the presence of small circular depressions (arrow) corresponding to Tomes’ processes of ameloblasts (A and B). Notice the presence of a single small defect of mild hypoplasia (arrow head) (B). (Original magnification: A - 2500X; B - 2500X)

FIG. (14) Scanning electron microscopy of the enamel surface of a rat’s mandibular incisor from the diabetic group showing mild hypoplasia with widespread superficial depressions (arrow) and a rough appearance. (Original magnification: 2500X)

FIG. (15) Scanning electron microscopy of the enamel surface showing moderate hypoplasia in a diabetic rat with superficial depressions of variable size and irregular form. Notice the appearance of the rod ends (dotted arrow) within the defects (A&B) and the presence of cracks (arrow) and small pores (arrow head) between the rod ends (B). (Original magnification: A - 2000X; B - 2500X).
DISCUSSION

In the search for a cell marker useful for studying tooth development, Tabata et al. [15] depended on the immunohistochemical studies using antibodies against cytokeratin 14 in the developing tooth of the newborn rat and in primary cultured cells of the ameloblast lineage, including inner enamel epithelium cells, preameloblasts and ameloblasts, prepared from the mandibular incisors of postnatal 7-day-old rats. The appearance of K14 was cell- and differentiation-stage specific, i.e. there was a weak expression signal within inner enamel epithelial cells that were in the proliferating stage, and there were strong signals within preameloblasts and ameloblasts that were in the post-proliferating and amelogenesis stages, respectively. In the culture system, K14 appeared mainly in clustered cells that were considered to be in the post-proliferating stage. These findings indicate that K14 is a good marker for ameloblast-lineage cells during rat tooth development both in vivo and in vitro. In accordance, in a study to grow human ameloblast-like epithelial cells in culture, DenBesten et al. [20] found that ameloblast-like cells were immunopositive for cytokeratin 14. Also Kawano et al. [21] reported that Immunoreactivity for CK14 was observed in the entire dental epithelium, and was especially intense in differentiated ameloblasts, outer enamel epithelium, and lingual epithelium. There was,

FIG. (16) Scanning electron microscopy of the enamel surface of a diabetic rat showing severe hypoplasia in the form of longitudinal defects (A and B) in which a deformed basket-weave pattern (arrow) could be seen (C). Very deep defects could be seen in some areas (D). (Original magnification: A - 200X; B - 1000X; C - 2500X; D - 5000X).
however, no reactivity observed in mesenchymal tissues of the incisor tooth germ.

The functions of intermediate filaments are poorly understood, but are presumed to be structural [22]. There is some evidence to suggest that cells may increase their intermediate filament content in response to mechanical loading [23,24]. Where coexpression of different intermediate filaments occurs, these cytoskeletal elements have been associated with the cell environment, cell shape or the possible secretory activity of the cell [25,26,27,28]. The specific distribution of different intermediate filaments indicates particular functions for these elements within tissues. It has been suggested that intermediate filaments could form part of a mechanotransduction system which enables the cells to respond to external forces and sense changes in the extracellular matrix [28]. It is proved that there is a specific interaction between K14 and amelogenin. It is also proposed that CK14 play a chaperon role for nascent amelogenin polypeptide during amelogenesis and mutations in K14 may suppress the secretion of amelogenin [29].

So the decrease in the staining intensity of ameloblasts for K14 in the present study could explain the detrimental influence of diabetes on the function of ameloblasts in laying down enamel.

Confocal laser scan microscopic observations on ameloblasts during postnatal (PN) growth of the teeth showed that the K5-amelogenin complex migrated from the cytoplasm to the periphery (on PN day 1) and accumulated at the apical region on day 3. Secretion of amelogenin commences from day 1. K5, similar to K14, may play a role of chaperone during secretion of amelogenin. Upon secretion of amelogenin, K5 pairs with K14. Pairing of K5 and K14 commences on day 3 and ends on day 9. The pairing of K5 and K14 marks the end of secretion of amelogenin [30].

In the present study the accumulation of K14 at the distal end of ameloblasts is some diabetic rats may indicate stagnated secretion.

In some skin diseases, defects in K5/K14 filament network architecture cause basal keratinocytes to become fragile and account for their trauma-induced rupture [31]. This may explain the rupture of some ameloblasts in the present study. As a previous study reported the presence of discolored and notched front teeth in a child with a functional Knockout of K14, displaying clinical symptoms of epidermolysis bullosa [20].

Enamel hypoplasia is the most common developmental defect of human teeth that may be seen in deciduous teeth of babies born to diabetic women. In their study on the enamel organ of the mandibular incisors of the offspring of rats with alloxan-induced diabetes, Silva-Sousa et al [1] observed thinning of the enamel matrix and of the ameloblasts and the nuclear area of the latter was smaller which agree with our results. In the rats born to treated diabetic mothers, the nuclei of the ameloblasts were more elliptical and there was enlargement of the interstitial area of the stellate reticulum. These results indicate that there are structural defects in the enamel organ of rats born to mothers with alloxan-induced diabetes which could induce the enamel hypoplasia which may reflect the metabolic alterations seen in this condition. Silva-Sousa et al [1] concluded that the severity of hypoplasia is variable and is correlated positively with the maternal level of blood glucose.

Millar et al [32] explained the loss of ameloblasts by the defective proliferation or differentiation of ameloblast precursors, progressive apoptosis of ameloblasts, or loss of ameloblast stem cells.

Karim [33] reported that the acute effect of a single injection of streptozotocin (75 mg/kg) on the secretory ameloblasts of rat incisor was found to be reversible after 4 h. The long term effect of streptozotocin was characterized mainly by large accumulations of secretion granules within the Golgi apparatus, the presence of many lysosomal structures, an abnormal redistribution of secretion granules, and the presence of large amounts of electron-dense material within the intercellular spaces. Because the electron-dense material resembled the enamel matrix, this, and the above changes, suggested
that there was a pronounced inhibition in the movement of secretion granules into Tomes’ process, as well as an ectopic secretion of the enamel matrix protein.

On the other hand, no differences of enamel microhardness values between diabetics and healthy persons, neither by longer diabetes duration nor by worse diabetes attitude have been found [34].

“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” [6].

REFERENCES


