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Healing process of incisor teeth of diabetic rats replanted after storage in milk

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Abstract – Several local factors that influence the healing process of replanted teeth have been investigated. However, it remains unclear how systemic alterations, such as diabetes mellitus, affect the prognosis of these cases. The purpose of this study was to evaluate the healing process of incisors of non-controlled diabetic rats replanted after storage in bovine long shelf-life (UHT) whole milk. Thirty-two rats were randomly assigned to receive an endovenous injection of either citrate buffer solution (group I – control; $n = 16$) or streptozotocin dissolved in citrate buffer solution to induce diabetes (group II; $n = 16$). After confirmation of the diabetic status by analysis of the glycemic levels, the maxillary right incisor of each animal was extracted and immersed in milk for 60 min. The root canals of teeth were then instrumented, and were filled with a calcium hydroxide-based dressing and replanted into their sockets. All animals received systemic antibiotic and were killed by anesthetic overdose 10 and 60 days after replantation. The specimens containing the replanted teeth were removed, fixed, decalcified, and embedded in paraffin. Semi-serial 6-μm-thick sections were obtained and stained with hematoxylin and eosin for histologic and histometric analyses. The results showed that the connective tissue adjacent to the root surface was less organized in the diabetic animals than in the control animals in both periods; the root dentin was less severely affected by root resorption in the diabetic rats; there were no significant differences between the control and diabetic groups regarding the occurrence of replacement resorption and inflammatory resorption.

Dental trauma is part of daily clinical practice and dentists must be prepared to deal with trauma cases and deliver the best care possible for each situation. Avulsion of permanent teeth is the most serious of all types of traumatic tooth injuries because the tooth is pulled out from its natural seat. The prognosis depends on the measures taken at the place of accident and/or the period immediately after the avulsion. Although immediate replantation is the treatment of choice, clinical experience has shown that most avulsed teeth are replanted after a long extra-alveolar time in dry or inadequate wet storage, which may lead to failure of replantation and tooth loss (1).

The success of tooth replantation depends, among other factors, on the maintenance of viable cemental periodontal ligament (PDL) cells and cementoblasts (2). Therefore, shortening as much as possible the time elapsed between trauma and replantation, and keeping the avulsed tooth in a suitable transport medium may attenuate the deleterious effects of the extra-buccal time on root surface and increase case prognosis (2–7). The substances frequently studied as storage media for exarticulated teeth include saline, saliva, water, milk, Hank's balanced salt solution, and Viaspan® (Belzer UW; CSS, Dupont Pharmaceuticals, Wilmington, DE, USA) (5–7). Milk has presented the best results among the storage media that are most commonly available at the site of accident (7, 8).

In case of necrotic tissue pulp or degenerated PDL acting as stimuli to inflammatory resorption, endodontic treatment (9) and systemic antibiotic therapy may be effective in preventing its occurrence or limiting its progression (10). However, little is known about the influence of systemic alterations, such as diabetes, on the healing of replanted teeth.

The term diabetes mellitus refers to a group of metabolic disorders that are characterized by hyperglycemia due to depleted secretion and/or action of insulin. Type 1 diabetes results from an autoimmune destruction of the insulin-producing pancreatic beta cells, causing an absolute deficiency in the production of this hormone. It comprises 5–10% of the cases and is more common in children and adolescents (11–13). Insulin plays a critical role in the regulation of blood glucose (14). Its deficiency in the body causes alterations in the metabolism of carbon hydrates, secondarily involving the protein and lipid metabolism (15). The persistence of high blood glucose levels may increase host's susceptibility to infections and cause a delay in wound healing (12, 16–19), including alveolar repair (12–14, 16–21).

The number of cases of diabetes worldwide in adults aged 20 years and older is currently estimated to be over 171 million (11). The high prevalence of obesity and physical inactivity allied to the world's population aging has led to a significant increase in the incidence of this disease in the last decades (22). As health professionals, dentists must be familiar with diabetes and its associated complications and must be prepared to deal with diabetic patients in different clinical situations. In case of severe traumatic injuries like tooth avulsions, the possible implications of diabetes in case management should be considered. The purpose of this study was to evaluate histologically and histometrically the healing process of incisors of non-controlled diabetic rats replanted after storage in bovine long shelf-life (UHT) whole milk.

Material and methods

The research project was independently reviewed and approved by the Animal Research Ethics Committee of the Dental School of Araçatuba, São Paulo State University/UNESP, Brazil.

Thirty-two adult male Wistar rats (*Rattus norvegicus albinus*) weighing approximately 250 g were selected for the study. The animals were housed in plastic cages under climate-controlled conditions (12 h light/12 h dark; thermostatically regulated room temperature) and were fed a standard solid chow (Ração Ativada Produtor; Anderson & Clayton S.A. Indústria e Comércio, São Paulo, SP, Brazil) and water *ad libitum*.

The animals received an intramuscular injection of xylazine hydrochloride (Coopazine; Coopers do Brasil Ltda., Cotia, SP, Brazil; 0.03 ml/100 g body weight) to attain muscular relaxation and were anesthetized intramuscularly with ketamine hydrochloride (Vetaset; Fort Dodge, São Paulo, SP, Brazil; 0.07 ml/100 g body weight). Thereafter, the rats were randomly assigned to two groups according to the treatment protocol: in group I (control; $n = 16$), the animals received an endovenous injection in the penile vein of citrate buffer solution 0.01 M, pH 4.5 (Farmácia Aphoticário; Araçatuba, SP, Brazil); in group II ($n = 16$), for experimental induction of diabetes, the animals received an endovenous injection in the penile vein of streptozotocin (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in citrate buffer solution at 35 mg kg^{-1} body weight. This concentration was used because it had provided an adequate glycemic level (> 350 mg dl^{-1}) in rats with 250 g body weight in a pilot study. Seven days after induction of diabetes, blood samples were collected from each animal's tail to determine the glycemic levels using an automatic blood glucose monitoring system (Accu-Chek Advantage; Roche Diagnóstica do Brasil Ltda., São Paulo, SP, Brazil).

After confirmation of hyperglycemia, the rats were anesthetized, the anterior maxilla was cleaned with iodine polyvinylpyrrolidone (Riodeine; Ind. Farmacêutica Rioquímica Ltda., Rio de Janeiro, RJ, Brazil) and the maxillary right incisor of each animal was extracted and immersed in 50 ml of bovine long shelf-life UHT whole milk (Parmalat Brasil S.A. Indústria de Alimentos, Santa Helena de Goiás, GO, Brazil) at room temperature (22°C) for 60 min. The teeth were then washed in saline (Ariston Ind. Química. e Farmacêutica. Ltda, São Paulo, SP, Brazil), the dental papilla and enamel organ were removed with a #15 scalpel blade (Embramac Exp. e Imp., Campinas, SP, Brazil) and the pulp tissue was extirpated through a retrograde route with a slightly curved #35 Hedström file (25 mm; Sybron Kerr Corporation, Orange, CA, USA). The canals were irrigated with saline (Ariston Ind. Quím. e Farmacêutica. Ltda), dried with absorbent paper points (Dentsply Ind. e Com. Ltda., Petrópolis, RJ, Brazil) and filled with a calcium hydroxide-based paste (5 g calcium hydroxide, 2 g zinc oxide, 0.015 g colophony, 5 ml polyethylene glycol; Farmácia Aphoticário), which was packed in empty sterile anesthetic cartridges and injected through retrograde route with a long curved blunt-tipped needle (G27; Terumo Corporation, Tokyo, Japan) attached to a Carpule syringe. The teeth were replanted in their respective sockets after gentle alveolar curettage and irrigation with saline. No type of retention was used. After replantation, all animals received a single intramuscular dose of benzathine G penicillin 20 000 IU (Benzetacil; Eurofarma Laboratórios Ltda, São Paulo, SP, Brazil).

The rats were killed by anesthetic overdose at 10 days ($n = 8$ per group) and 60 days ($n = 8$ per group) after replantation. The right maxilla containing the replanted tooth was separated from the left maxilla at the midline and a cut was made with straight scissors on the region distal to the third molar The specimens containing the replanted teeth were removed, fixed in 10% buffered formalin (Dinâmica Reagentes Analíticos, São Paulo, SP, Brazil) for 24 h and decalcified in 4.13% EDTA solution, pH 7.0. After decalcification, the specimens were embedded in paraffin and longitudinal semi-serial 6-μm-thick sections were obtained (one microscopic slide per specimen) and stained with hematoxylin and eosin for histologic and histometric analyses.

Histologic analysis

The images of the longitudinal root sections were first divided into three thirds (cervical, middle, and apical) using a compass, a ruler, and a fine pen. The region corresponding to the middle root third was analyzed under a light microscope for determining the characteristics of the PDL, cementum, dentin and alveolar bone, as well as the occurrence of superficial, inflammatory and replacement resorption, and ankylosis. Only the middle third of the lingual surface of the roots was examined histologically and histometrically because this region is not damaged by the surgical procedures. The cervical third may be damaged by the grasping action of the forceps during tooth extraction while the apical third may be damaged by the cutting action of the scalpel blade during dental papilla removal.

Histometric analysis

For histometric measurements of the resorbed root surface area, sections of both groups obtained at the 60-day period were analyzed. The images were captured

using a digital video camera (JVC TK-1270 Color Video Camera, Tokyo, Japan) coupled to a Carl Zeiss microscope (Axiolab, Thornwood, NY, USA) and connected to a computer. Two image captures (720 × 480 pixels) were made to cover the entire middle third. The images were saved as individual TIFF files and joined at ×200 zoom using Corel Photo Paint 12 image-editing software (Corel Corporation, Ottawa, ON, Canada). Care was taken to standardize the size and position of the areas examined in all sections.

The images were analyzed using ImageLab® 2001 image-analysis software (Diracom Bio Informática, Vargem Grande do Sul, SP, Brazil). The area of resorbed root dentin in each specimen was demarcated (in pixels) and the non-resorbed root dentin area was obtained by subtracting the area with inflammatory and/or replacement resorption from the total root dentin area. The severity of root resorption in the groups was analyzed statistically by determining the amount of non-resorbed dentin. Statistical calculations were performed using SAS software version 8.02 (SAS Institute Inc., Cary, NC, USA). As data distribution was normal, one-way ANOVA was used for analysis of the non-resorbed dentin area in each group at the 5% significance level.

Inflammatory and replacement root resorption data were also analyzed statistically. However, a different approach was adopted in this case because resorption was not observed in several specimens, producing a zero value that could not be used for statistical calculations. Therefore, the absolute values were transformed into percentage and a 4-point scoring system was used as follows: 1 = no resorption; 2 = 0.1–50% of the area with resorption; 3 = 51–99% of the area with resorption; 4 = 100% of the area with resorption. As data distribution was non-normal, the nonparametric Mann–Whitney test was used at the 5% significance level.

Results

The animals endured adequately all experimental procedures. One animal from group II (diabetes) was removed from the study due to root fracture during tooth extraction, which reduced the number of animals to seven in the 60-day period.

10 Days

Group I (Control; n = *8)*
In most specimens, cementum and dentin were intact, except for few regions that presented areas of superficial resorption. In some of these areas, an inflammatory infiltrate with multinucleate cells was observed close to the resorbed dentin (Fig. 1). The PDL was rich in collagen fibers, fibroblasts, and newly formed blood vessels. The collagen fibers were thin and did not have a well arranged organization. In several specimens, an inflammatory cell infiltrate with a moderate number of lymphocytes and few macrophages was also observed in some areas. Thin bone trabeculae that did not reach the cementum surface were found in this region. The presence of several osteoblasts and resorbed areas with osteoclasts in the alveolar bone indicated an ongoing remodeling activity.

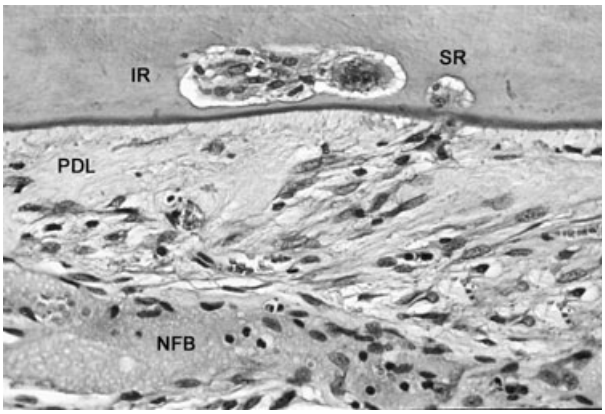

Fig. 1. Group I (Control rats) – 10 days. Area of superficial resorption (SR) and inflammatory resorption (IR) with multinucleate cells. Periodontal ligament (PDL) remnants with fibroblast proliferation and newly formed bone tissue (NFB). HE ×250.

Group II (Diabetic rats; n = *8)*
Intact cementum and dentin were observed in almost all examined roots. Few areas of superficial resorption were observed. Some areas of inflammatory resorption exhibited multinucleate cells (Fig. 2). The PDL was rich in collagen fibers, fibroblasts, and newly formed blood vessels and, in most specimens, PDL remnants were present close to the root surface. Compared to the control group, the connective tissue presented a smaller fibroblast proliferation and less organized collagen fibers close to the alveolar bone walls. Moderate lymphocyte and macrophage infiltrate was noted more frequently in this group (Fig. 2). In two specimens, polymorphonuclear neutrophils were also found in some areas of degenerated connective tissue. Thin bone trabeculae that did not reach the cementum surface were found in this region. Similar to that observed in the control group,

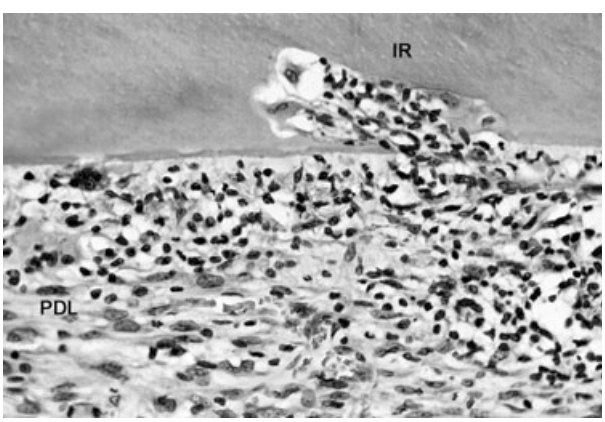

Fig. 2. Group II (Diabetic rats) – 10 days. Area of inflammatory resorption (IR) and periodontal ligament (PDL) space with fibroblast and capillary proliferation. Presence of inflammatory lymphocyte infiltrate with few macrophages. HE ×250.

areas of resorption with multinucleate cells were observed in the alveolar bone wall.

60 Days

Group I (control; n = *8)*
Only two specimens presented inflammatory root resorption, involving few areas of the root surface, which were filled by fibrous connective tissue with a lymphocyte and macrophage infiltrate. Areas of replacement resorption were found in seven specimens, in which the cementum and the resorbed dentin were replaced by bone tissue. In two of these specimens, replacement resorption involved more than 70% of the root surface while in the other five specimens, less than 30% of root surface was affected. In four of these specimens, the root surface exhibited an intact cementum layer and fibrous connective tissue involving almost the entire examined area (Fig. 3). The PDL space was occupied by collagen fibers, a large number of fibroblasts, and a moderate number of lymphocytes and macrophages. In several areas, collagen fiber organization suggested reattachment. In some areas, multinucleate cells were observed close to the border, facing the alveolar bone trabeculae. In few areas, thin bone trabeculae were found joined to the alveolar walls and root surface.

Group II (Diabetic rats; n = *7)*
Only two specimens presented inflammatory root resorption involving the small cementum and dentin areas. In three specimens, replacement resorption reached few areas of the cementum and dentin (Fig. 4). In these specimens, most part of the root surface was intact and covered by a fibrous connective tissue. Only one specimen of this group presented a small ankylosed area in which the alveolar bone was juxtaposed to the cementum surface, filling the PDL space completely. In four specimens, the cementum and dentin were also intact, but areas of degenerated connective tissue were found close to the root surface. In this region, the collagen fibers appeared disorganized near the alveolar bone wall.

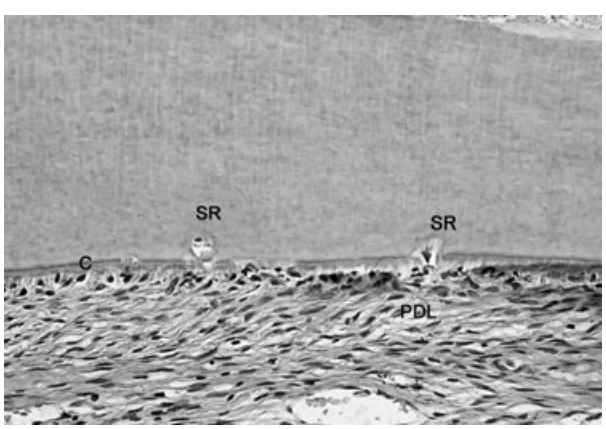

Fig. 3. Group I (Control rats) – 60 days. Root surface with intact cementum layer (C) and reattachment of periodontal ligament (PDL) fibers. Areas of superficial resorption (SR). HE ×63.

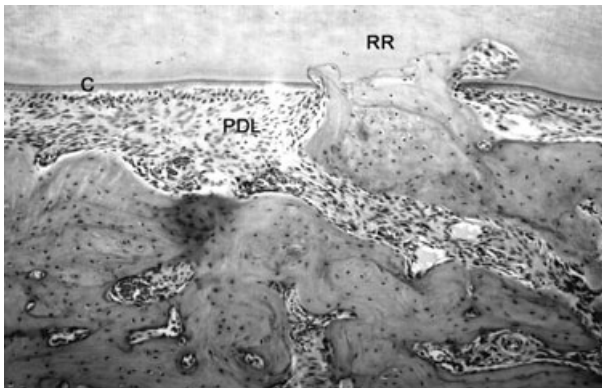

Fig. 4. Group I (Diabetes rats) – 60 days. Area with intact cementum layer (C) and periodontal ligament (PDL) with large number of fibroblasts and collagen fibers. Replacement resorption area (RR) on root surface. HE ×63.

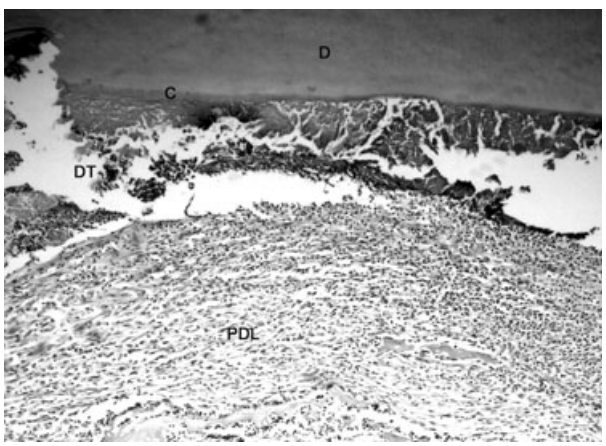

Fig. 5. Group II (Diabetic rats) – 60 days. Intact cementum (C) and dentin (D) and presence of degenerated tissue (DT) in contact with the cementum. Disorganized periodontal ligament (PDL) collagen fibers. HE ×63.

A moderate inflammatory cell infiltrate was also observed in this area (Fig. 5).

Statistical analysis

The diabetic group presented a significantly larger area of non-resorbed dentin compared with the control group at the 60-day postoperative period (Table 1).

Regarding the scores attributed to inflammatory and replacement resorption, there was no statistically

Table 1. Number of teeth, means (in pixels), and standard deviations among the groups regarding the area of non-resorbed dentin

Groups	No. teeth	Means	SD	*P*
Control	8	3760.28	1736.03	0.0323*
Diabetes	7	7468.12	4901.73	0.1260

*Statistically significant ($P < 0.05$).

Table 2. Frequency of scores among the groups regarding inflammatory resorption and replacement resorption

	Inflammatory resorption		Replacement resorption	
Scores	Control group	Diabetes group	Control group	Diabetes group
1	6	5	1	4
2	2	2	5	3
3			2	
4				
n	8	7	8	7

Statistically significant at 5%.

significant difference ($P > 0.05$) between the control group and the diabetic group at the 60-day postoperative period (Table 2).

Discussion

In the present study, streptozotocin was used for experimental induction of diabetes because it reduces the synthesis of nicotinamide adenine dinucleotide (23), causing the destruction of pancreatic beta cells (24, 25) and consequently inhibiting the production of insulin (26, 27). This systemic condition may be confirmed 1 h after endovenous administration of streptozotocin (27). Injection of streptozotocin via the penile vein presented as a technically more favorable procedure (28).

Clinical signs of polydipsia, polyuria, polyphagia, and weight loss, typically found in diabetes (12–14), were observed in the animals of group II during the course of the experiment. Although this condition manifested 24 h after streptozotocin injection (28), determination of glycemic levels (14, 29) and tooth extraction were performed only 7 days after induction of diabetes, respectively, in order to allow adaptation of the animals to the new clinical status.

Histometric and statistical analyses were performed only with the specimens from the 60-day period because more significant alterations were found at that time. Replantation was performed 60 min following extraction because most avulsed teeth are replanted after this extra-alveolar time in clinical dental practice (4, 30). The bovine long shelf-life (UHT) milk used in this study as a storage medium was maintained at room temperature because it is more likely to be available non-refrigerated at the moment of accident. However, it has been demonstrated that a more favorable prognosis is expected when exarticulated teeth are kept in refrigerated transport media (3, 31). According to Lekic et al. (31), temperatures closer to 4°C produce significantly better results than room temperature.

Despite the use of milk as a storage medium in both groups, disorganized collagen fibers and inflammatory cell elements were more frequent in the diabetic rats in the earlier period. This result could be attributed to the delay in the initial inflammatory response caused by diabetes (19, 20, 32). At 60 days, the connective tissue was less organized and mature in some specimens, which demonstrates that diabetes interfered in the proliferation and function of fibroblasts and collagen fiber metabolism. The occurrence of these alterations in diabetic organisms has previously been reported by other authors in different types of experiments (15, 19, 20, 33).

Three types of root resorption may occur, depending on the PDL and pulp tissue conditions (34). Superficial resorption occurs when small areas of the PDL and cementum are damaged. Inflammatory resorption results from the presence of contaminants in the PDL remnants and/or pulp tissue. If contaminants are not present and the damaged area is extensive, ankylosis or replacement resorption can occur (35). In the present study, endodontic treatment with use of a calcium hydroxide-based dressing (9) and systemic antibiotic therapy (10) were effective in controlling inflammatory resorption, as demonstrated by the low occurrence of this type of resorption in both groups. Similar findings have been reported in a previous study (36).

The administration of a systemic antibiotic had an important role because non-controlled diabetes is associated with an increase in the frequency and severity of oral infections (13, 18, 37). Yet, areas of necrotic tissue in the region corresponding to the PDL were found in two specimens in the earlier period. These findings suggest that the host's immune response might have been compromised. The immune system shows unfavorable reactions in cell and humoral activities in the diabetic status. The opsonization capacity of the blood cells is abnormal, and phagocytosis is the part of the cell system that most reflects this abnormality (19, 38).

The areas of resorbed root dentin were smaller in the diabetic group, which may also be due to the delay in the healing process caused by diabetes (20). Diabetes has a deleterious action on chemotaxis, proliferation and activity of the phagocitary cells responsible for resorption (17, 30, 38, 39), and also delays the inflammatory response (19, 20).

The histologic findings of this study suggest that indication of tooth replantation in patients with non-controlled type 1 diabetes should be done with care. The areas of degenerated fibrous connective tissue observed in few specimens and the possible progression and involvement of a larger root surface area by inflammatory resorption should also be considered (40, 41). Nevertheless, a greater concern would be the possible contamination of this tissue, which could aggravate the hyperglycemic status (39). Further research would contribute to support the basis for indication of tooth replantation under the conditions proposed in this study.

Conclusions

According to the proposed methodology and based on the results of the present study, it may be concluded that: (i) The connective tissue adjacent to the root surface of the diabetic animals was less organized than that of the control animals in both periods; (ii) The root dentin of the diabetic rats was less severely affected by root resorption; (iii) There was no significant difference between the control and diabetic groups regarding the occurrence of replacement and inflammatory resorption.

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