Effect of Propolis on Induced Alveolar Bone Loss in Diabetic Rats

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Abstract:
Introduction: Periodontitis is the 6th classic complication of Diabetes Mellitus (DM). Both Diabetes & periodontitis are chronic inflammatory diseases. As DM accelerates bone resorption; when periodontal disease affects a diabetic individual, extensive alveolar bone loss occurs. Propolis is a natural antibiotic & anti-inflammatory agent that honey bees (Apis mellifera) collect from tree buds or other botanical sources; and use in the construction as well as the protection of their hives. Due to its broad pharmacological potential, propolis has been employed extensively in medicine, since ancient times.

Objective: Was to evaluate the effect of systemic administration of propolis on the alveolar bone loss, induced by periodontitis, in diabetic rats.

Materials and methods: Twenty-four Albino rats were divided into three equal groups: group (1) normal control (NC), group (2) Diabetes + Periodontitis (DP) and group (3) Diabetes + Periodontitis + Propolis (DP-Pro). DM type 1 was induced in rats of groups (2) and (3), by single intraperitoneal injection of Streptozotocin. Then, periodontitis was induced by ligature placement sub marginally around the right mandibular 1st molar. In group (3), propolis was administrated systemically by gastric feeding 400mg/Kg/day for 8 weeks. At the end of this period, animals were sacrificed and dissected. The alveolar bone surface integrity interproximally was evaluated by scanning electron microscopy (SEM), and results were compared between groups.

Results: After 2 months, group (2) showed more alveolar bone loss compared to the 2 other groups. Groups (1) & (3) showed an intact, smooth & regular bone surface, while group (2) specimens showed an irregular & porous bone surface with extensive resorption.

Conclusion: This study showed that the systemic administration of propolis effectively prevented the extensive alveolar bone loss associated with experimental periodontitis in diabetic rat models. Thus, propolis might be used as an adjunctive treatment for periodontitis associated with Diabetes.

Keywords: Propolis, Diabetes Mellitus, Alveolar bone loss, Periodontitis.

INTRODUCTION

Bone is a living dynamic tissue, which makes up the body skeleton and is one of the hardest structures of the human body (1). The adult skeleton is broken down continuously and reformed; this replacement of old bone by new is called "bone turnover" or "remodeling"(2). This is performed by clusters of bone resorbing osteoclasts and bone forming osteoblasts.

In a healthy individual, this turnover is in a steady state; so that, the amount of bone lost is balanced by formed bone (2). The status of the bone represents the net result of a balance between the two processes (1). A decreased bone formation and/or increased bone resorption results/result in bone loss. In certain diseases (e.g., osteoporosis) and with age, the resorption exceeds formation; leading to an overall bone loss (2). The alveolar bone is that portion of maxilla and mandible which surrounds and supports roots of teeth. Resorption of the alveolar bone and other supporting structures of the tooth causes tooth loss (1).

Diabetes Mellitus (DM) is a chronic systemic metabolic disorder (3). It is characterized by hyperglycemia due to a deficiency of insulin secretion caused by pancreatic β-cell dysfunction and/or insulin resistance (3). DM causes morbidity and mortality as its long-term complications affect many important organs(3).

Clinical complications of DM include retinopathy, nephropathy, neuropathy, macro-vascular disease, delayed wound healing, and periodontal disease(4).

In November 2014, the World Health Organization (WHO) stated that the burden of diabetes is increasing globally, particularly in developing countries; mainly, due to obesity and physical inactivity (5). The International Diabetes Federation (IDF) reported that the worldwide prevalence of diabetes was 8.5% in 2014. 387 million people are living with diabetes and the number is expected to rise to 205 million by 2035(6).

In addition to the complications mentioned above, diabetes affects bone metabolism as it accelerates bone resorption. Animal studies have demonstrated that alveolar bone loss in rats with Streptozotocin (STZ)-induced type 1 Diabetes Mellitus (TIDM) with periodontitis was threefold higher than in normal rats(7). Today, chronic periodontitis has been identified as the sixth complication of diabetes (5).

Periodontitis is one of the most widespread inflammatory diseases, characterized by periodontal pocket formation, clinical attachment loss, and alveolar bone destruction(8). The major component of both soft and hard tissue destruction in periodontitis occurs as a result of the
hyperactivation of the host immune-inflammatory response against pathogenic bacterial plaque (9).

Several mechanisms have been proposed to explain the great incidence and severity of periodontal disease in diabetics (10). These include increased susceptibility to infection due to diminished neutrophil recruitment and altered function, as well as, the effect of advanced glycation end products (10). The latter can include enhanced formation of inflammatory cytokines and delayed wound healing leading to increased tissue destruction (10, 11). In general, these mechanisms lead to increased formation of osteoclasts and bone loss (12).

Propolis is a natural resinous mixture that honey bees (Apis mellifera) collect from living plants, buds, and trees mixed with products of their salivary glands, wax, and pollen (13). Propolis is a Greek word, where "Pro" stands for "at the entrance of" or "before" and "polis" stands for "community" or "city" which means that this natural product is used in the defense of the hive. It's also called "bee glue" (13).

Honeybees use propolis in the construction of their hives, repair and sealing openings and cracks to make a barrier against external invaders like snakes, lizards, weathering threats like wind and rain (13). The primary function of propolis in the hive is to act as a biocide, being active against invasive bacteria, fungi, and Invading larvae; making the beehive one of the most steril environments known to mankind (14).

Raw propolis is composed of resin (50-55%), essential oils, wax (30%), pollen (5%), and other organic compounds (5-10%) such as amino acids, minerals, vitamins (A, B complex, E) and the highly active biochemical substance known as bioflavonoid (15). Sugars are thought to be introduced accidentally during the elaboration of propolis and/or passage of bees over the resin (13).

Propolis has been employed extensively since ancient times. It was already known in ancient Egypt, where Egyptians benefited from the anti-putrefactive properties of propolis in order to mummify their pharaohs; also as an adhesive (13). The Arabs knew probably also about propolis. Doctor Avicenna (Ibn-Sinā) spoke of two different kinds of wax: clean wax and black wax, the latter being probably propolis (14).

Propolis was also known to the old Greeks; it was mentioned by the Greek philosopher and scientist Aristotle in his Historia animalium (16). Hippocrates, the founder of western medicine, used it for healing sores and ulcers internally and externally (15). Propolis was used as an antiseptic and cicatrizing agent by the Greek and Roman physicians. Propolis became very popular in Europe between the 17th and 20th centuries due to its antibacterial activity; it was listed as an official drug in the London pharmacopoeias of the 17th century (13).

Propolis has received great attention due to its biological and pharmacological properties which include anti-inflammatory (17), immunomodulatory (18), antibacterial (17), antioxidant (13) and anti-diabetic (19). It has been found recently that propolis induces bone healing (20). It also inhibits receptor activator of nuclear factor kappa beta, (NF-kB), ligand (RANKL) induced osteoclastogenesis & osteoclasts maturation in cell culture (21).

Nowadays, propolis is a natural remedy found in many health food stores, it is also used in cosmetics or as popular alternative medicine for self-treatment of various diseases (13). Current applications of propolis include formulations for cold syndrome (upper respiratory tract infections, common cold, and flu-like infections), as well as dermatological preparations useful in wound healing, treatment of burns, acne, herpes simplex and genitalis, and neurodermatitis (13). It is commercially available in the form of capsules, creams, throat lozenges, powder and also in many purified products from which the wax was removed (13). Propolis is also used in mouthwashes and toothpastes to prevent caries and to treat gingivitis and stomatitis (13).

The aim of the present study was to evaluate the effect of Propolis on alveolar bone loss, induced by periodontitis, in diabetic rats.

**MATERIALS AND METHODS**

**Animals & experimental design:**
The Ethical committee of the Faculty of Dentistry Alexandria University approved the protocol of this research. This experimental study was performed on rat models. Twenty-four male Albino rats, weighting 250-300 grams (approximately 6-7 months of age), were selected from the Institute of Medical Research Alexandria University. They were checked to have a full set of teeth. The rat is a monophydont animal with a dental formula as follows: I 1/1, C 0/0, P 0/0, M 3/3. Animals were housed in specially designed wire mesh bottom cages, four per cage; and were supplied a regular diet and water during the whole experimental period. Optimal conditions and good ventilation were maintained.

Rats were divided randomly into 3 groups, 8 rats each: Group (1): Normal control (NC) group, where rats were injected with vehicle (citrate buffer) Group (2): Diabetes + Periodontitis (DP) group. Group (3): Diabetes + Periodontitis + Propolis (DP-Pro); DM & periodontitis were induced in animals of groups (2) and (3); and in the latter rats received a daily dose of propolis 400mg/kg for 2 months.

**Materials:**

**Propolis**
Pure propolis was obtained in the form of Bio Propolis (Sigma pharmaceutical industries for IBE pharma) capsules 400mg each. The content of each capsule is in the form of water soluble propolis powder.
Streptozotocin (STZ)(22, 23):
Streptozocin (Sigma-Aldrich) or Izostazin or Zanosar is an antibiotic derived from Streptomyces achoromenes and structurally is a glucosamine derivative of nitrosourea. It is a synthetic antineoplastic agent and anti-tumor; used in cancer chemotherapy(23). A single injection > 40mg/kg exerts a diabetogenic effect through a specific damage of the pancreatic islet insulin-producing β-cells, mimicking type 1 DM in medical research(22). Pure STZ has an alkaline pH; however, it is stable as a solution around pH of 4. Thus, it should be freshly prepared in cold citrate buffer (pH 4.5) to enhance stability immediately before injection (23).

Procedures performed in study groups:
Induction of Diabetes Mellitus type 1: (22, 24, 25)
Rats in groups (2) & (3) were used as experimental models for induction of Diabetes Mellitus type 1, by single intraperitoneal (i.p.) injection of STZ 60mg/kg body weight freshly mixed with 0.1M citrate buffer (PH 4.5)(24).

In rats control group (1) were injected with vehicle (equal amount of citrate buffer) to control the influence of any injection stress or buffer-induced effects on animals(24).

Measurement of blood glucose level: (25-27)
Diabetes was confirmed in the study groups by analysis of blood glucose after an overnight fast, 48 h after STZ injection (25). Blood samples were obtained from tail vein of the rats according to the institutional animal care and use committee (IACUC) guidelines approved in 2/2015(26). Then, their blood glucose level was determined in mg/dl using digital Glucometer and glucometer test strips (OneTouch® Select®) according to the corresponding user guide. Rats with fasting blood glucose level above 135mg/dl or postprandial blood glucose above 190mg/dl was considered diabetic (27).

Induction of periodontitis: (25)
After an overnight fast, the rats in groups (2) & (3) were anesthetized with ketamine (1ml/kg i.p.) & Xylazine (0.1ml/kg i.p.) for sub gingival placement of 4.0 silk ligatures around 1st mandibular molar. Ligatures were knotted submarginally, by the same operator, around the gingival margin using a needle holder. Ligatures were placed after confirmation of diabetes induction. The ligatures were kept in place during the experimental period and served as retentive factor for dental plaque.

Administration of propolis: (28)
Each rat in group (3) received 400mg/kg/day for 8 weeks(28). Propolis powder was dissolved in water, well agitated, and then administrated by intra-gastric feeding using oral gavage.

Scanning Electron Microscopic (SEM) examination: (29, 30)
By the end of the 8th week, the animals were sacrificed. The mandible of each rat was dissected, and separated from muscle and soft tissue, keeping the attached gingiva intact with the bone. The mandible was split into halves from the midline between the central incisors. Only the segment of the mandibular molar teeth with the surrounding alveolar bone was then prepared for SEM. Specimens were fixed in 2.5% glutaraldehyde in phosphate buffer (PH 7.3) for 48 hours and washed twice in the same buffer. The specimens were then dehydrated in the graded series of aqueous ethanol solution 50%, 70%, 90%, and 100% for one hour for each specimen. Then they were air-dried, mounted on aluminum SEM stubs with silver paint and sputter coated with gold using an ion coater (sputter coater)(29). The specimens were examined by SEM using a 20-kV accelerating voltage; to observe the alveolar bone surface integrity buccally using magnifications X500 & X3500.

The alveolar bone was judged based on the smoothness of its surface; normally bone shows a smooth surface penetrated by nutritive canals, resorption is indicated by irregularities, pits and depressions as osteoclasts form Howship's lacunae (30).

RESULTS:
Results of the scanning electron microscope of the bone surfaces showed differences among the different groups. However, all specimens of the same group showed similar results. In addition, the buccal cortical plates showed the same morphological features from the apical to the cervical area.

Results of SEM in control group (1) showed:
An intact alveolar crest, in the interproximal area between 1st & 2nd molars, without any destruction (Fig. 1A). The surface topography of the buccal cortical plate of alveolar bone was smooth, regular, and uniform. The cortical bone surface revealed regularly bordered and well defined nutritive canals (Fig. 1B).

Group (2) showed:
Alveolar bone destruction was noted and the surface topography exhibited a generalized pattern of surface porosity, roughening and irregularities (Fig. 2A). The resorbed bone surface showed scalloped pits and craters revealing the active osteoclastic bone resorption; less defined nutritive canals having irregular borders (Fig. 2B).

Group (3) DP-Pro showed:
No alveolar bone destruction, the alveolar crest was restored to normal architecture (Fig. 3A).
Fig. (1A): Scanning electron micrograph (SEM), group (1), showing: Intact buccal cortical plate (BC) and alveolar crest (arrow) between 1st & 2nd molars (M1 & M2 respectively). (X500)

Fig. (1B): SEM, group (1), showing: Smooth, uniform, and homogenous buccal cortical plate (BC) surface is with a regularly bordered nutritive canal (dotted arrow). (X3500)

Fig. (2A): SEM, group (2), showing: Alveolar crest (arrow) between 1st and 2nd molars (M1 & M2 respectively) with severe resorption and destruction. (X500)

Fig. (2B): SEM, group (2), buccal cortical plate (BC) showing: Rough, irregular porous & osteoporotic bone surface filled with scalloped pits and craters (dotted arrows). (X3500)
DISCUSSION:
Diabetes has been shown to enhance osteoclast formation in inflamed areas (31). This was confirmed in several studies; rats with T1DM and periodontitis also exhibit a two- to four-fold increase in the number of osteoclasts compared to non-diabetic rats with periodontitis (32).

In addition, Diabetes impairs the resolution of periodontal inflammation. A higher degree of inflammation and a more persistent inflammatory response following periodontitis are reported in rats with T1DM and T2DM (33). Similarly, patients with periodontitis and diabetes were found to have significantly higher levels of local inflammatory mediators such as Interleukin 1b (IL-1b), Tumor necrosis factor alpha (TNF-α), and prostaglandin E2, which result in more prolonged osteoclast formation and activity (34).

In this study, we evaluated the effect of propolis (400mg/kg/day for 2 months) on alveolar bone loss induced by periodontitis in a diabetic rat model for the first time using SEM.

Streptozotocin (STZ) 69% and alloxan (31%) are by far the most frequently used drugs for chemical induction of (DM) (35). In the present study, STZ was used because it is a simple, effective and available method (23). Streptozotocin is a DNA alkylating agent that enters cells exclusively via the glucose transporter 2 (GLUT2) proteins, resulting in rapid and irreversible necrosis (35).

Several models of experimental periodontitis, such as dietary manipulation, introduction of pathogenic microorganisms, and placement of a ligature, have been described in the literature (36). Ligature method was chosen, as it has been accepted as a useful experimental model of periodontitis with alveolar bone resorption; in addition it promotes scenarios similar to food impaction between teeth & bacterial accumulation (36). However, this condition is different from chronic periodontitis in humans, due to its acute course of inflammation, which is induced by tissue trauma during placement (25). Moreover, molars in rats are similar in anatomic configuration and structure to those in humans, but the molars of rats are smaller, so it was difficult to perform any type of periodontal treatment (37).

In this study, both diabetes and periodontitis caused severe alveolar bone destruction in (DP) group compared to (NC) group; these results are similar to those of Kim et al.(7), who found upon histological examination, that T1DM aggravates alveolar bone loss induced by periodontitis. Kim et al. (38) as well found that in ligatured teeth, alveolar bone loss was increased in both STZ and STZ- nicotine amide (STZ-NA)-treated rats compared to control rats using histomorphometric analysis.

Pontes Andersen et al. (39), in a study investigating the effect of ligature-induced periodontitis on prediabetic state in Zucker fatty rats (ZFRs), showed that ZFRs with periodontitis demonstrated significantly more bone loss compared to lean rats with periodontitis; and concluded
that Prediabetes is a predisposing factor for periodontitis. Liu et al. (40) also found that diabetes caused greater alveolar bone resorption, and impaired new bone formation. Pontes Andersen et al. (41) reported in another study, after 6 weeks of induced periodontitis, that long-term bone loss in type 2 diabetic Goto–Kakizaki rats was significantly greater than controls.

On the other hand, the administration of propolis in the present study, prevented alveolar bone loss & restored its normal original architecture. Aral et al. (25) found similar results using Histomorphometric measurements, the propolis group showed significant reduction in alveolar bone loss. In a study performed by Toker et al. (36) propolis, when administered systemically, prevented alveolar bone loss in the rat model of experimental periodontitis.

In a study performed on human beings, Sanghani et al. (17) revealed that the subgingival delivery of propolis is promising as an adjunct to scaling and root planing (SRP) in patients with chronic periodontitis; when assessed by clinical and microbiological parameters According to Ang et al. (21) Caffeic acid phenethyl ester (CAPE), one of the active components of propolis, can prevent bone loss in cell cultures through the suppression of cell signaling pathways of RANKL induced nuclear factor kappa beta (NF-κβ) and Nuclear Factor of Activated T-Cell (NFAT) activity. Al-Molla et al. (42) revealed that coating of implants with propolis showed increased osseointegration in short interval period.

CONCLUSION:
The findings of this study provide evidence that propolis, when administered systemically, prevents periodontitis induced alveolar bone loss in diabetic rats. So, it may be used as adjunct treatment besides conventional treatment of periodontitis, especially in diabetic patients.

CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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blood pressure and interstitial pH in early developmental stage of insulin resistance in OLETF rats by intake of propolis extracts. Biochemical and biophysical research communications.


