ABSTRACT

BACKGROUND: Diabetes mellitus (DM) is one of the most common endocrine disorders. It is associated with an elevation in blood glucose levels. Oxidative stress caused by hyperglycemia is central to the occurrence of diabetic complications such as retinopathy, nephropathy and diabetic osteopenia. Alveolar Bone forms the primary support structure of teeth. It’s affected majorly by diabetic osteopenia. Some antidiabetic drugs may also increase the risk of diabetic fractures. Hesperidin is an active flavanone isolated from citrus fruits.

OBJECTIVES: The aim of the current study is to evaluate the effect of Hesperidin administration on alveolar bone structure in rats with Streptozotocin-induced diabetes.

MATERIALS AND METHODS: Eighteen adult male albino rats will be randomly divided into 3 equal groups: Group I (Control group). Group II (DM group): Rats will be administered Streptozotocin (STZ) to induce DM. Group III (Hesperidin group): Rats with STZ–induced DM will be given Hesperidin via gastric gavage. Rats will be euthanized after 6 weeks. Mandibles will be dissected and prepared for histological evaluation by light and scanning electron microscope. Percentages of Calcium and Phosphorus will be analyzed using EDX.

RESULTS: Control group showed normal histological features of the alveolar bone. The diabetic group revealed an irregular outline with severe destruction while the Hesperidin group showed almost normal restoration of the structure of alveolar bone.

CONCLUSION: Hesperidin exerted a protective effect on alveolar bone osteoporosis in diabetic rats.

KEYWORDS: Hesperidin, Diabetes Mellitus, alveolar bone, osteoporosis.

INTRODUCTION

Diabetes mellitus (DM) is considered to be one of the most common endocrinial disorders affecting millions around the globe. It is a compelling public health issue in both developing and developed countries. It is a complex chronic metabolic condition with a global incidence of 451 million individuals in 2017 and a projected increase to 693 million by 2045. It is becoming more prevalent because of population expansion, ageing, urbanization, and an increase in obesity and physical inactivity (1). Diabetes Mellitus is characterised by abnormal lipid and carbohydrate metabolism, glucose intolerance, and increased blood glucose levels (2).

Hyperglycemia is induced by a lack of insulin production, which is mediated by pancreatic β-cell deterioration or by blocking of peripheral receptors to insulin action in the liver and muscle, or by a union of the two (3).

Diabetes’ persistent hyperglycemia is linked to long-term harm, malfunction, and breakdown of various organs, particularly the kidneys, eyes, heart, nerves and bone. This is referred to as “Diabetic Complications.” Among these complications are nephropathy, neuropathy, retinopathy, end stage renal disease, and osteopenia (4).

Osteopenia, in particular, is always linked to a higher bone fracture frequency, a delay in bone fracture repair, alterations in bone mineral content and a diminishing in diabetic patients’ quality of living (5).

Hyperglycemia leads to reactive oxygen species (ROS) generation which is the prime motive of oxidative stress in diabetes (6). ROS weaken cellular antioxidant means of defence, making the damaged cells and tissues more vulnerable to oxidative damage. Inflammation induced by the increase in ROS intracellularly is also related to diabetic complications. Therefore, managing hyperglycemia and oxidative stress at the same time has a high potential for decreasing the occurrence and progression of diabetic osteopenia (7).

Flavonoids are polyphenolic compounds that occur naturally in vegetables and fruits. They own a large variety of biological properties, including antiviral, antibacterial, immune-
stimulant, anticancer, and antioxidant capabilities (8).

Many citrus flavonoids have been demonstrated to lower oxidative stress, strengthen insulin sensitivity and glucose tolerance, suppress inflammation and apoptosis, control lipid metabolism and adipocyte differentiation, and enhance endothelial malfunction, suggesting a possible anti-diabetic effect (9).

Hesperidin (HESP) is a significant dynamic flavanone glycoside whose chemical formula is “5,7,3-trihydroxy-4-methoxy-flavanone 7-rhamnoglucoside” and it is obtained mostly from citrus fruits. HESP has further medical benefits due to its antibacterial, antiallergic, radioprotective, and antioxidant characteristics (10).

In humans, hesperidin was shown to lower blood pressure and boost antioxidant status. This citrus flavanone is a widely employed treatment used to cure or prevent circulatory system disturbances (lowering capillary permeability), as well as an anti-carcinogenic, anti-inflammatory herbal medication (11).

Hesperidin reduced serum insulin and blood glucose levels in an animal study of STZ-induced diabetes. It also restored the enzymatic reactions of glucose-6-phosphatase which is involved in glycemic regulation (12).

Regarding its effect on bone, Hesperidin was found to reduce femur bone loss in 2 month old ovariectomized mice when 0.5 % Hesp was given in their diets (13).

In young and adult intact rats, Hesperidin was also found to prevent ovariectomized-induced femoral bone loss. (14) Since studies evaluating the effect of hesperidin on alveolar bone structure are limited, this study was done to determine its effectiveness on diabetes-associated osteoporosis.

The null hypothesis in this study assumes that oral administration of hesperidin has no significant effect on alveolar bone structure in rats with induced diabetes.

**MATERIALS AND METHODS**

This study design was accepted by the Ethical Committee of Faculty of Dentistry, Alexandria University. The Approval number by the ethical committee is 0218 and IORG 0008839.

**Study sample**

Eighteen adult male albino rats 6 months old (200-250 grams in weight) were utilized in this study. Animals were acquired from the animal house of Medical Research Institute, Alexandria University. They were held at the same environmental conditions in the experimental animal house.

The light microscope procedures of the study were done in Oral Biology department, Faculty of Dentistry, Alexandria University. The scanning electron microscope procedures of the study and EDX analysis were done in Faculty of Science, Alexandria University.

**Grouping (Randomization technique)**

Rats were randomly assigned by (using computer generated random numbers) into three groups (6 rats for each group):

**Group I (Control group):** Healthy rats were injected with saline.

**Group II (Diabetes Mellitus group):** Rats were administered Streptozotocin dissolved in citrate buffer intraperitoneally at a single dosage of 50 mg/kg. (15)

**Group III (Hesperidin group):** Rats with Streptozotocin-induced DM were given Hesperidin at 200 mg/kg/day orally via gastric gavage for 6 weeks. (16)

**Induction of Type I Diabetes Mellitus (15)**

Rats of groups II and III were injected intraperitoneally with a freshly prepared solution of Streptozotocin dissolved in citrate buffer with pH 4.5 at a dosage of 50 mg/kg after 12 h of fasting. Diabetes was confirmed in rats of groups II and III if tail blood glucose concentrations were greater than 200-300 mg/dl using a glucometer in fed rats 2-4 days following STZ injection. Streptozotocin induces the apoptosis of Langerhans islet β-cells, resulting in experimental diabetes in 2-4 days (17).

**Administration of Hesperidin**

Hesperidin which was used in this experiment is available in powder form and was dissolved in 1mL of 0.9% saline and given orally to group III at 200mg/kg/day using gastric gavage, 2 days after induction of diabetes for a period of 6 weeks. (15, 16, 18)

**Euthanasia of experimental animals**

Rats were euthanized after six weeks by decapitation. The mandible of each rat was dissected out and divided into 2 halves. The right halves were prepared for light microscopic examination, while the left halves were prepared for Scanning Electron Microscopy and Energy Dispersive X-ray microanalysis. Method of disposal of the rats was done by burning.

**Light microscope procedures**

The right mandibular molar segments were tagged and preserved in 10% neutral buffered formalin with the surrounding alveolar bone. After fixation, specimens were decalcified with 8% trichloroacetic acid, washed, dehydrated in escalating ethanol concentrations, cleared with xylene, penetrated and imbedded in paraffin wax. Using a rotary microtome, thin sections of 5 µm thickness were sliced mesiodistally. For the purpose of histological examination, sections were then stained with Hematoxylin and Eosin (H&E) (19).

**Scanning Electron Microscope Procedures**

The left mandibular molar segments with the surrounding alveolar bone were separated and were immediately fixed in 4%FIG solution in phosphate buffer (PH 7.2) and thoroughly washed.
in the very same buffer. After that, the specimens were desiccated in a graded sequence of aqueous ethanol solutions. Then they were air-dried and exposed to critical point drying. Eventually, they were placed on aluminium stubs and gold sputtered with a sputter coater. The surface characteristics of alveolar bone in the three different groups was examined using scanning electron microscopy (20).

Statistical Analysis

The EDX microanalysis results were analyzed and processed using the One way ANOVA test to examine the overall differences between the three groups. Pairwise comparison between each 2 groups was done using Post Hoc Test.

RESULTS

Histological results:

Group I (control group)
The results obtained from the control group revealed the normal architecture of the alveolar bone with a regular outline. A continuous layer of active plump osteoblast cells was seen lining the bone surface. Osteocytes were regularly distributed with normal nuclear size. Incremental lines were seen indicating bone remodeling. The cancellous bone showed normal bone trabeculae. Bone Marrow tissue showed normal cellularity and vascularity. (Figures 1A and 1B)

Group II (Diabetes Mellitus group)
Examination of alveolar bone revealed a punched out irregular outline with severe destruction. Discontinuity of the osteoblast cell layer was seen. Osteocytes were noted having pyknotic nuclei while some lacunae appeared empty. Deeply stained reversal lines were seen indicating a high degree of bone remodeling. The cancellous bone related to the middle region revealed thin and irregular bony trabeculae associated with severe widening of bone marrow spaces. Bone marrow tissue showed abundant inflammatory cell infiltrate. (Figures 2A and 2B)

Group III (Hesperidin group):
Light microscopic findings of Hesperidin group showed an almost generalized restoration of the of the alveolar bone with a normal and regular outline. The osteoblastic layer restored its continuity along the bone surface, showing active plump osteoblast cells. Osteocytes showed normal density and distribution, with majority having normal nuclear size. The cancellous bone restored its regular and normal trabeculation enclosing the bone marrow spaces. Normal cellularity and vascularity of the bone marrow tissue was also evident. (Figures 3A and 3B)

Scanning electron microscope (SEM) results:

Group I (control group)
The surface topography of the alveolar bone's buccal cortical plate revealed an overall smooth and homogeneous surface. It also showed multiple nutritive canals with regular borders. (Figure 4)

Group II (Diabetes Mellitus group):
The surface topography of the buccal cortical plate revealed marked generalized roughness with irregular resorptive craters, porosities and prevailing deep areas of erosions and pits. Irregular nutritive canals surrounded by roughly resorbed borders were also observed. (Figure 5)

Group III (Hesperidin group):
The surface of the buccal cortical plate exhibited generalized uniform, smooth and homogenous pattern free of defects very close to the architecture as in the control group. The alveolar bone surface revealed marked masking of all resorptive changes related to diabetic group. Intact well-defined nutritive canals with regular borders were also noticed. (Figure 6)

Energy dispersive x-ray analysis (EDX)
Means, medians, and standard deviations are used to summarise calcium and phosphorous levels in the three groups. The diabetic group (group II) had a statistically significant reduction in calcium levels and a rise in phosphorous levels in relation to control group (P1 for calcium < 0.001) and (P1 for phosphorous < 0.001). On the other hand, in the hesperidin group (group III), the results weren’t statistically significant in comparison to the control group as the levels of calcium and phosphorous were much closer to the control group (group I) than that of diabetic group (P2 for calcium = 0.751) and (P2 for phosphorous = 0.507). Moreover, there was a substantial statistical difference in calcium and phosphorous levels between the hesperidin and diabetic groups (groups III and II, respectively) (P3 for calcium < 0.001) and (P3 for phosphorous < 0.001). (Tables 1 and 2)
evident. Bone marrow tissue show normal cellularity and vascularity. D: Dentin, C: Cementum, PDL: Periodontal ligament. (H&E x100) B: The cancellous bony region showing dense bone trabeculae surrounding normal cellular and vascular bone marrow spaces. Note the presence of several normal sized, well oriented osteocytes. (H&E x100)

Figure 2: LM (Diabetic group), A: The middle region of the alveolar bone showing severe resorption and an irregular outline. Discontinuity of the osteoblastic cell layer can also be seen (arrows). Widening of the osteocytic lacunae with pyknotic nuclei of osteocytes (arrowheads). Thinner bone trabeculae are noted. D: Dentin, C: Cementum, PDL: Periodontal ligament. (H&E x100). B: The cancellous bone region showing severe widening of the bone marrow spaces containing abundant inflammatory cell infiltrate (arrowheads). Note thinning of the bony trabeculae (arrows). BMS: Bone marrow space. (H&E x100)

Figure 3: LM (Hesperidin group), A: The middle region of the alveolar bone showing a regular bone outline. Note the layer of plump osteoblast cells (arrowheads). Osteocytes show normal size and distribution (Dashed arrows). Deeply stained reversal lines can be seen indicating bone remodeling (arrows). (H&E x100). B: The cancellous bony region showing normal organization of the bony trabeculae surrounding the bone marrow tissue. Bone marrow spaces are lined by endosteal cells (arrowheads) and they show normal cellularity and vascularity. Parallel resting lines can be seen (arrows). (H&E x100).

Figure 4: SEM (Control group) showing a generalized smooth bone surface with a nutrient canal having regular and smooth borders. (x1000)

Figure 5: SEM (Diabetic group) showing a deep resorptive crater in the center with marked generalized roughness and resorption of the bone cortical plate. (x1000)

Figure 6: SEM (Hesperidin group) showing marked masking of almost all resorptive changes in the cortical plate with a nutrient canal having a regular outline. (x1000)

Table 1: Comparison between the three studied groups according to calcium level.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=8)</th>
<th>Diabetes Milletus group - 6 weeks (n=8)</th>
<th>Hesperidin group -6 weeks (n=8)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (Min. - Max.)</td>
<td>26.4 (21.2 - 29.6)</td>
<td>10.7 (6.5 - 13.7)</td>
<td>25.3 (19.6 - 27.7)</td>
<td>51.586</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean ± SD.</td>
<td>25.7 ± 3.2</td>
<td>10.7 ± 3.7</td>
<td>24.5 ± 2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. bet. grps</td>
<td>p1&lt;0.001* , p2=0.751</td>
<td>p2&lt;0.001*</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

SD: Standard deviation
F: F for One way ANOVA test, pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three studied groups

- p1: p value for comparing between Control and Diabetes Mellitus group - 6 weeks
- p2: p value for comparing between Control and Hesperidin group - 6 weeks
- p3: p value for comparing between Diabetes Mellitus group - 6 weeks and Hesperidin group - 6 weeks

*: Statistically significant at p ≤ 0.05

Table (2): Comparison between the three studied groups according to Phosphorus

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=8)</th>
<th>Diabetes Mellitus group - 6 weeks (n=8)</th>
<th>Hesperidin group - 6 weeks (n=8)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media (Min - Max.)</td>
<td>12.7 (9.8 - 15.9)</td>
<td>7.7 (4.8 - 10.9)</td>
<td>14.3 (12.1 - 15.2)</td>
<td>29.502</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean ± SD.</td>
<td>12.9 ± 1.9</td>
<td>7.5 ± 2.2</td>
<td>13.9 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. bet. grps</td>
<td>p1&lt;0.001*, p2&lt;0.507, p3&lt;0.001*</td>
<td></td>
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</table>

SD: Standard deviation

DISCUSSION
Diabetes is known as a group of metabolic disorders defined by hyperglycemia caused due to abnormalities in insulin production, insulin action, or both. Diabetes mellitus (DM) is linked to a variety of microvascular and macrovascular consequences. (22) Diabetes-induced osteoporosis (DOP) is a chronic metabolic bone illness known by increased osteoclast activity, higher amounts of advanced glycation end products (AGEs) and reduced osteoblast activity (23).

The principal support structure for the teeth is the alveolar bone. All bone tissues share the cellular functions and factors engaged in the creation and remodelling of alveolar bone. (24) Hesperidin is a flavanone glycoside that is obtained mainly from citrus fruits. The majority of this bioflavonoid's therapeutic effects has been linked to its capability to regulate pro-inflammatory cytokines including TNF, IL-1, and IL-6, as well as decrease inflammation and oxidative stress. (11)

This study evaluated the effect of Hesperidin, when given orally at a dose of 200mg/kg, on alveolar bone structure in a diabetic rat model. This was done histologically and by SEM. Percentages of Calcium and phosphorus were also analyzed using EDX.

Diabetes mellitus was induced in experimental animal models using a variety of methods such as chemical, surgical (pancreatectomy), and genetic changes. Streptozotocin and alloxan are by far the most commonly utilized agents for chemical stimulation of diabetes. Both medications have a diabetogenic effect when taken parenterally (intravenously, intraperitoneally or subcutaneously) (25).

The animal model chosen in this experiment was rats due to their anatomical and genetic similarities to humans. They are also chosen because they are readily available, inexpensive with a well-defined skeleton. (26) The reduced lifespan of rodents also allows for the study of ageing in bone metabolism and regeneration processes. In addition, rodents are used for in vivo testing of therapeutic approaches to the bone tissue. (27)

In the present study, STZ was given at a dosage of 50 mg/kg per body weight. This is compatible with the findings of Shehata et al. who used the same dose to develop diabetes in rats (15). The dose that has been observed to cause the most diabetic conditions in rats is 50 to 75 mg/kg Ip (intraperitoneal). STZ-induced diabetes is not persistent at lower dosages because spontaneous recovery occurs (28).

Histological results of the control group in the current study revealed normal architecture of the alveolar bone with plump osteoblast lining and osteocytes showing normal size and distribution. These results concur with those of Lima et al. (29) who studied the anti-resorptive effects of Calendula Officinalis on inflammatory alveolar bone loss in rats and described the normal architecture of the alveolar bone.

On the other hand, the diabetic group showed a punched out irregular outline of the alveolar bone. Deeply stained reversal lines were noted indicating increased bone remodeling. These observations are in conformity with Qi et al. (30) who reported that Type 1 diabetic rats have lower bone mineral density, which leads to bone fragility. He added that elevated blood glucose levels,
Hesperidin administered intragastrically effectively reduced ligature-induced alveolar bone resorption in rats. Hesperidin reduced gingival inflammation and inflammatory marker expression, demonstrating the anti-inflammatory activity of hesperidin.

Scanning electron microscope (SEM) results supported the histological findings as it showed that the control group revealed a regular and smooth surface topography. Unlike the diabetic group which showed surface roughness with resorptive craters and porosities, indicating diabetic osteoporosis. Wang et al. (38) assessed the structure of diabetic mouse femurs using micro-CT scanning, histomorphometry, and radiographic imaging. In diabetic mice, trabecular bone density and thickness were considerably lower than in the control group. He then concluded that Bone Morphogenetic protein 6 can reduce Type 1 diabetes-related osteoporosis.

However, SEM results of the Hesperidin group showed an almost uniform and smooth cortical plate free of defects, very close to the control group. Chiba et al. (39) also found that Hesp reduced osteoporosis caused by Androgen insufficiency in male mice using bone histomorphometric indices such as BV (Bone Volume)/TV (Trabecular Volume) and Trabecular Number. (Tb. N.)

In diabetic mice, EDX demonstrated a reduction in calcium concentration in respect to phosphorous. The EDX conclusions were consistent with SEM results. According to Prati et al., EDX can be used to compute element ratios and analyse the mineralization degree of bone, as well as the content of mineralized tissues or mineral (apatite) deposits. A lot of researchers have employed the calcium to phosphorous ratio to assess bone mineralization (40).

The results of EDX of the Hesperidin group revealed an increase in the calcium level and the relative restoration of the normal calcium-phosphorus ratio in relation to the diabetic group. Zhang et al. (35) suggested that Hesperidin’s anti-osteoporotic effect in OVX-induced osteoporotic rats is due its ability to maintain Ca and P homeostasis. The proper balance of Ca and P levels is essential for maintaining healthy bone and supporting bone development.

**CONCLUSION**

Diabetes-induced osteoporosis, which is a metabolic bone disease, is caused mainly by hyperglycemia and increased oxidative stress. Hesperidin was found to exert protective effect on alveolar bone osteoporosis in diabetic rats. This is mainly due to its potent osteogenic effect.

**CONFLICT OF INTEREST**

The authors report that they have no conflict of interest.

**FUNDING**
This study did not collect any specific funds.

REFERENCES


