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The ability of hesperidin compared to that of insulin for preventing osteoporosis induced by type I diabetes in young male albino rats: A histological and biochemical study

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ABSTRACT

Background: Patients with type I diabetes are at increased risk of osteoporosis even after insulin therapy in adult stage. This study was conducted to compare the efficacy of hesperidin (hesp) therapy versus that of insulin alone in the alleviation of osteoporosis arising from type I diabetes mellitus (T1DM) in young rats.

Materials and methods: Hesperidin was administered orally to STZ-induced diabetes. The animals were evaluated morphologically and biochemically and compared with that received daily SC injections of long-acting insulin.

Results: Histologically, we observed the degeneration of osteoblasts and osteocytes, decreased collagen fibers, and disturbed bone turn over markers in untreated DM rats. Hesperidin+ insulin supplementation to diabetic rats caused significant improvement of most of the bone histological and morphometric parameters compared with the insulin-treated group. Furthermore, hesp treatment significantly reduced pro-inflammatory mediators TNFα and NF-κB and increased serum biochemical markers of bone turnover, including osteopontin (OPN), osteocalcin (OC) and decreased serum alkaline phosphatase (ALP).

Conclusion: These data demonstrated that hesp could be considered to be a beneficial drug for preventing diabetic osteoporosis in growing age.

1. Introduction

Diabetes is one of the most important metabolic disorders in the world that causes death, lifelong disability and complications (Gispen and Biessels, 2000). Insulinopaenia in T1DM is associated with osteoporosis (Nicodemus and Folsom, 2001). Type 1 diabetic patients are at high risk for bone loss, bone fracture and delayed fracture healing with significantly lower bone mineral density (Heap et al., 2004; Bayat et al., 2013). Disastrous outcomes could be associated with simple fracture in diabetic patients even after insulin treatment (Mann et al., 2010; Birdee and Gloria, 2010).

Oxidative stress has been implicated in pathogenesis of diabetes-related bone diseases. In recent years, many studies demonstrated potential efficacies of polyphenols on metabolic disorders and complications induced by diabetes. Flavonoids are present in several types of vegetables and fruits, and may be associated with potential health benefits (Sharma et al., 2008; Cao et al., 2007).

Hesperidin (hesp) is considered as a citrus bioflavonoid exclusively found in citrus fruits and called sometimes “Vitamin P”. It acts as an antioxidant and proved by other investigator to have anti-cancer and anti-inflammatory because of its antioxidative activities (Mahmoud et al., 2012).

Hesperidin acts on bone to increase the anabolic process and increase bone production (Lister et al., 2007). Studies published in the last few years have shown that citrus compounds may promote bone health. Although the hypoglycemic and hypolipidemic effects of hesperidin in rodents are well studied, the actual molecular mechanism of this effect is not well established (Akiyama et al., 2010). They act early in life when maximal bone mass is reached

Abbreviations: ALP, Alkaline phosphatase; BTM, bone turnover marker; BW, Body weight; DM, Diabetes Mellitus; Hesp, Hesperidin; H&E, Hematoxylin & Eosin; OPN, Osteopontin; OC, Osteocalcin; STZ, Streptozotocin; T1DM, Type 1 diabetes mellitus.

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and later in life during post-menopausal osteoporosis (Martin et al., 2016). Many studies detected beneficial role of hesp on postmenopausal osteoporosis (Chiba et al., 2003; Martin et al., 2016).

Therefore, the aim of this study was to investigate whether hesperidin can be an effective regimen for ameliorating bone complications in growing rats with diabetes and comparing the results with that receiving long acting insulin.

2. Materials and methods

2.1. Chemical and drugs

Streptozotocin (STZ, Cat # S0130) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lantus (insulin glargine) was manufactured by Sanofi–Aventis (Frankfurt am Main, Germany). Hesperidin (Cat # H5254) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Eighty male albino rats aged 3 weeks (just after weaning; 70 ± 12 g body weight) were included in the study. The rats were fed a laboratory pellet diet and provided tap water ad libitum. The animals were left for two weeks accommodation before start of the experiment.

The experiment was performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources, 1996) approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Zagazig University, Egypt.

2.3. Animal grouping

Group I (control): included 20 rats randomly assigned from initial 80 rats. They were treated intraperitoneally with vehicle only.

The rest 60 animals received STZ and then randomly assigned to three equal groups. Group II (non-treated DM): The animals with diabetes were untreated.

Group III (insulin-treated DM) and group IV (insulin+ hesp-treated DM): The animals with diabetes were administered daily SC injections consisting of 2 units of long-acting insulin (Kohzaki et al., 2008). In addition, animals of group IV were administered a daily dose of hesperidin at a dose of 200 mg/kg by orogastric tube (Bang et al., 2012).

Another group of animals was used to detect the effect of hesp on normal bone tissues to be sure that this dose is optimal and not causing any changes in normal tissue. No significant changes were detected at biochemical or structure level between this group and control group. Data are not shown.

2.4. Induction of diabetes

For induction of diabetes, rats of groups II, III, and IV were administered a single freshly prepared intraperitoneal injection of STZ dissolved in citrate buffer (pH 4.5) at a dose of 50 mg/kg after 12 h of fasting (Lee, 2006). The rats were have diabetes if the tail blood glucose concentrations were greater than 200–300 mg/dl using a glucometer in fed animals 2 days after STZ injection (Frode and Medeiros, 2008). Tail vein blood glucose samples from the groups with diabetes (II, III, IV) were measured weekly with a glucometer to observe blood glucose levels throughout the experiment.

2.5. Sampling and histological analysis

The general health conditions of the rats were observed and recorded daily. Body weights of all animals were measured weekly and at the end of the experiment.

At the time of sacrifice (8 weeks after the induction of diabetes), the animals were anesthetized. Fasting blood samples (after 12–14 h) were withdrawn from the retro-orbital vein and processed immediately into two tubes. The first tube contained fluoride for the immediate estimation of fasting blood glucose, and the second tube was allowed to clot at room temperature and then centrifuged. The cleared sera were separated into small glass tubes and stored at $-20\,^{\circ}C$ prior to the biochemical analysis.

The femurs were carefully dissected and cleared from the adjacent muscles. Parts of the femurs were snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ to measure TNFα and NF-κB tissue mRNA expression. The other part was decalcified according to the method described by Bancroft and Gamble, 2002.

For the light microscopy study, the decalcified femurs were processed and stained with haematoxylin and eosin (H&E) for routine histological analysis and Masson trichrome to assess the collagen fibers. For the transmission electron microscopy study, small specimens of the decalcified diaphysis were processed according to the method of Glauret and Lewis, 1998. Ultrathin sections were stained and examined with a JEOL-JEM 1010 electron microscope in the histology department of the Faculty of medicine, Zagazig University, Egypt.

2.6. Morphometric analysis

Sections of the femurs from all groups were examined under a light microscope at 400× magnification. Measurements were taken from 10 non-overlapping fields of each specimen using an automatic analysis system and the Leica image analysis software. The number of osteoblasts was recorded; Osteoblasts were identified as single-nucleated, rod shaped cells attached to bone surface.

The mean cortical bone thickness (μm) was measured by drawing vertical lines from just beneath the periosteum to the endosteum (Balena et al., 1993). The coloured area percentage of the blue-stained collagen fibers was measured in the trichrome-stained sections.

Each field was scanned together with a microscale by a "Leica Quin" image analyser computer system (Leica Imaging System Ltd., Cambridge, England). The measuring frame of a standard area is equal to 7286, 78 μm^2. The same method was applied for measuring area percentage of the blue coloured areas for collagen fibers in Masson's trichrome stained sections but the measuring frame was of area 118476, 6 and an objective lens 100 in 10 fields for each specimen.

2.7. Biochemical analysis

2.7.1. Serum alkaline phosphatase (ALP) estimation

Serum alkaline phosphatase (ALP) estimation was measured as an enzyme marker of osteoblasts using a commercial kit (Bio-Diagnostics. Dokki, Giza, Egypt).

2.7.2. Serum osteopontin (OPN) and osteocalcin (OC)

Serum osteopontin (OPN) and osteocalcin (OC) were measured using a rat osteopontin assay kit (Immuno-Biological-Laboratories, Gunma, Japan) and a rat osteocalcin IRMA kit (Immunotopics, San Clemente, CA, USA), respectively.

2.7.3. Quantitative estimation of serum insulin

Quantitative estimation of serum insulin was performed using a rat insulin ELISA kit (Mercodia, USA).

2.7.4. Quantitative real-time PCR

Total RNA was prepared from the proximal femur using a commercial kit (RNeasy Mini Kit, QIAGEN GmbH, Hilden, Germany) after washing out bone marrow cells and homogenizing the tissue in the presence of 0.1 M EDTA.

Real-time PCR was performed with the QuantiTect SYBR Green RT-PCR master mix (QIAGEN GmbH, Hilden, Germany) and specific primers (Table 1) using the StepOne™ System (Applied Biosystems). The PCR reactions were performed in final volumes of 25 μl. Briefly, the reactions consisted of 12.5 μl of 2× QuantiTect SYBR Green RT-PCR master mix, 12.5 μl of 2× QuantiTect SYBR Green RT-PCR master mix, 0.25 μl of QuantiTect RT mix, the indicated concentrations of primers, and template RNA. The cycling conditions involved reverse transcription at 50 °C for 30 min and enzyme activation at 95 °C for 15 min. The amplification program consisted of 1 cycle for 1 min at 95 °C, followed by 45 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Each RT-PCR sample was run in duplicate. The mean CT values were calculated. Target gene expression was normalized to the housekeeping gene β-actin. The $2-\Delta\Delta Ct$ method was applied to calculate relative gene expression. The primers used for RT-PCR for TNFα, NF-κB and β actin are provided in Table 1.

2.8. Statistical analysis

Data were expressed as the means ± SD. The statistical analysis was performed using the SPSS statistical program version 17. Data were evaluated using one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (least significant different test; LSD). The data were considered significant when P was less than 0.05.

3. Results

3.1. General results and body weight (BW)

At the end of the experiment, the untreated group with diabetes had significantly decreased BWs (Table 3) accompanied by lethargy, drowsiness, and polyurea. Abscess formation was noted in some of the untreated group (5 rats); the abscesses were treated topically. Groups that have diabetes and treated with hesp +insulin or insulin alone showed a good general condition. Significant elevation was recorded in the BWs of hesp +insulin -treated DM group compared to the untreated DM and insulin- treated DM groups.

3.2. Histological results

3.2.1. Detection of general bone architecture by H&E-stained sections

Control rats showed normal compact bone of the shaft of the femur. Outer and inner circumferential bone lamellae are regular giving smooth surface to the bone. Interstitial bone lamellae are regularly organized formed of Osteocytes embedded in their lacunae with large nuclei together with Haversian canals containing blood vessels (Fig. 1a).

Compact bone of untreated diabetic group was degenerated. Apparent decreased bone thickness and degenerated osteocytes are seen. Osteocytes were shrunken with pyknotic nuclei. The endosteum appeared irregular with multiple notches (Fig. 1b). There were some empty lacunae devoid of cells and multiple erosion cavities containing osteoclasts with acidophilic cytoplasm and multiple nuclei (Fig. 1c). Sections from the insulin-treated DM rats showed relatively large areas of immature bone; these areas contained more cells and the matrix was not layered in osteonal arrays. Osteocytes with pyknotic nuclei and erosive cavities were seen (Fig. 1d).

Compact bone sections from the hesp +insulin -treated DM showed nearly normal architecture, more cells and a less layered matrix in osteonal arrays were observed. Most of the osteocytes were normal but a few cells were degenerated. There were basophilic cement lines indicating bone repair. The outer and inner bone surfaces appeared regular (Fig. 1e).

3.2.2. Detection of collagen fibers in bone matrix by Masson's trichrome-stained sections

Lamellar arrangement of abundant blue-stained collagen fibers was apparent in the control group (Fig. 2a). Diabetes caused thinning of the cortex and a marked decrease in the demonstrated collagen (Fig. 2b). Treatment with insulin alone exhibited a relative decrease in collagen and a loss of its lamellar arrangement (Fig. 2c). Preservation of distribution of collagen fibers was maintained by adding hesp to insulin in diabetic rats (Fig. 2d). The red-stained osteoid (bone organic matrix) in all groups was inversely proportional to the amount of collagen.

3.2.3. Ultrastructure of osteoblasts

Control osteoblasts were aligned along the growing surfaces of the bone and surrounded by a small amount of osteoid-containing collagen fibrils. Each cell contained a euchromatic nucleus with little peripheral heterochromatin, Golgi saccules, plenty of rough endoplasmic reticulum (rER) and short cytoplasmic processes (Fig. 3a). In contrast, osteoblasts of the untreated DM group were elongated, had reduced cytoplasmic area contained dense nuclei with irregular outlines and a vacuolated cytoplasm with little or absent rER profiles. Interstitial space was wide filled with collagen fibers (Fig. 3b). Treatment with insulin alone failed to restore normal osteoblasts. They still elongated with some vacuoles and a non-prominent rER profile (Fig. 3c). In contrast, the hesp +insulin -treated DM group showed active osteoblasts with plenty rER (Fig. 3d).

3.2.4. Ultrastructure of osteocytes

The control group revealed osteocytes with stellate shapes residing in lacunae. The osteocytes showed nuclei and cytoplasmic canalliculi. The surrounding matrix contained abundant collagen fibrils (Fig. 4a). Osteocytes from the untreated DM group were shrunken with dense nuclei and blunt cytoplasmic processes. Their lacunae was large and lucent. The matrix around the cells was rarefied (Fig. 4c and d). Some osteocytes from the insulin treated DM group showed dense nuclei and blunt cell processes and the surrounding matrix was rarefied (Fig. 4e and f). Osteocytes from the hesp +insulin -treated DM group contained stellate cells trapped in lacunae and surrounded by an organized matrix (Fig. 4g and h).

3.3. Histomorphometric results (Table 2)

The mean number of osteoblasts in the non-treated DM groups was significantly decreased ($P < 0.05$) compared with the control group and the other groups. A significant increase in the mean

Table 1
Primer sequence and expected product size of genes amplified.

	Primer sequence	Fragment size
TNFα	F: 5-TCT CAA GCC TCA AGT AAC AAG C-3 R: 5-ATG AGG TAA AGC CCG TCA GC-3	310 bp
NFκB	F: 5-TAC CAT GCT GTT TTG GTT AC-3 R: 5-TCA AGC TAC CAA TGA CTT TC-3	208 bp
β actin	F: 5-TGT TGT CCC TGT ATG CCT CT-3 R: 5-TAA TGT CAC GCA CGA TTT CC-3	306 bp

Table 2
Comparison between of the osteoblast, osteoclasts number and area percentage of collagen fibers among the studied groups. Data are represented as mean ± SD.

Groups	Cortical bone thickness (μm)	the number of osteoblasts	Number of osteoclasts	area% of collagen in the femur
Group I (control)	368.1 ± 27.5	12.1 ± 2.8		1579.13 ± 85.09
Group II (non- treated DM)	293.1 ± 21.7[a,b,c]	4.9 ± 3.8[a,b,c]		1122.23 ± 132.02[a,b,c]
Group III (DM + Insulin)	349.1 ± 14.9[a,c,d]	8.3 ± 3.4[a,c,d]		1327.51 ± 131.95[c,d]
Group IV (DM + Hesp)	369.27 ± 17.8[a,b,d]	11.6 ± 2.7[b,d]		1523.17 ± 79.09[b,d]

[a] Significantly different from group I.
[b] Significantly different from group III.
[c] Significantly different from group IV.
[d] Significantly different from group II.

Table 3
Mean ± SD of body weight, serum level of glucose, osteopontin, osteocalcin and alkaline phosphatase in all studied groups.

	Control Group I	Diabetes Group II	Diabetes + Insulin Group III	Diabetes + Hespirdin Group IV
Body weight (g)	220.5 ± 2.3	110 ± 3.5[a,b,c]	210 ± 15.66[a,c,d]	228.1 ± 8.1[b,d]
Food intake (g/day)	11.68 ± 0.28	14.50 ± 0.21[a,b,c]	11.71 ± 0.4[d]	11.80 ± 0.1[d]
Serum glucose mg/dl	83.7 ± 6.5[d]	349.6 ± 77.8[a,b,c]	90.4 ± 6.2[d]	85.6 ± 7.1[d]
Osteopontinng/ml	46.1 ± 2.9[d]	39.2 ± 2.6[a,b,c]	41.9 ± 2.5[a,c,d]	44.7 ± 2.5[b,d]
Osteocalcinng/ml	13.8 ± 2.7[d]	6.7 ± 1.2[a,b,c]	10.1 ± 2.3[a,c,d]	12.7 ± 2.4[b,d]
Alkaline phosphatase U\L	84.9 ± 9.5[d]	194.8 ± 36.4[a,b,c]	98.1 ± 9.5[a,c,d]	80.4 ± 10.8[b,d]
Serum insulin ng/dl	1.79 ± 0.09[d]	0.26 ± 0.04[a,b,c]	1.82 ± 0.01[d]	1.72 ± 0.09[d]

[a] Significantly different from group I.
[b] Significantly different from group III.
[c] Significantly different from group IV.
[d] Significantly different from group II.

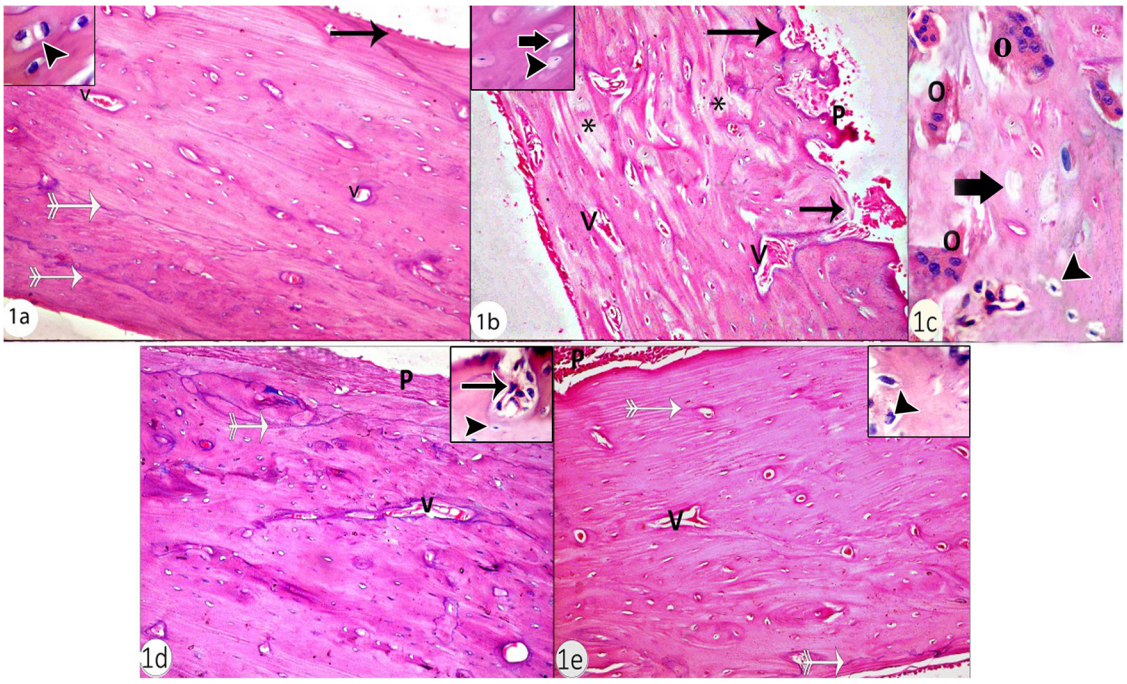

Fig. 1. H&E-stained sections of the shaft of the femur. (1a): Control group showing cortical bone lamellae with Haversian canals (v) containing blood vessels and smooth endosteal surfaces lined with cuboidal osteoblasts (arrow). Subperiosteal bone deposition appears as distinct basophilic cement lines (white arrows). Inset: showing normal osteocytes trapped inside lacunae (arrow head) (1b): Untreated diabetic bone showing thinning of the shaft, focal areas of the bone matrix appear homogenous and faint, lack of fibers (*), and the irregularly eroded endosteum (P) with a notched surface (arrow). Some lacunae appear empty and devoid of osteocytes (thick arrow), some osteocytes contain small pyknotic nuclei (arrow heads). (1c): multiple erosion cavities containing osteoclasts (o) housed in the eroded bone surfaces. (1d): Diabetic + insulin treated rats showing relatively less layered matrix and more cells. Cement lines (white arrows), periosteum (P) are also seen. Inset: showing erosion cavity (arrow) on the endosteal surface of the bone containing osteoclasts and osteocytic lacuna with pyknotic nuclei (arrow head) (1e): Diabetic group rats treated with hesp +insulin showing nearly normal compact bone architecture with osteoblasts on the bone surfaces (P). Inset: normal osteocytes in their lacunae (arrow head). H&E ×400.

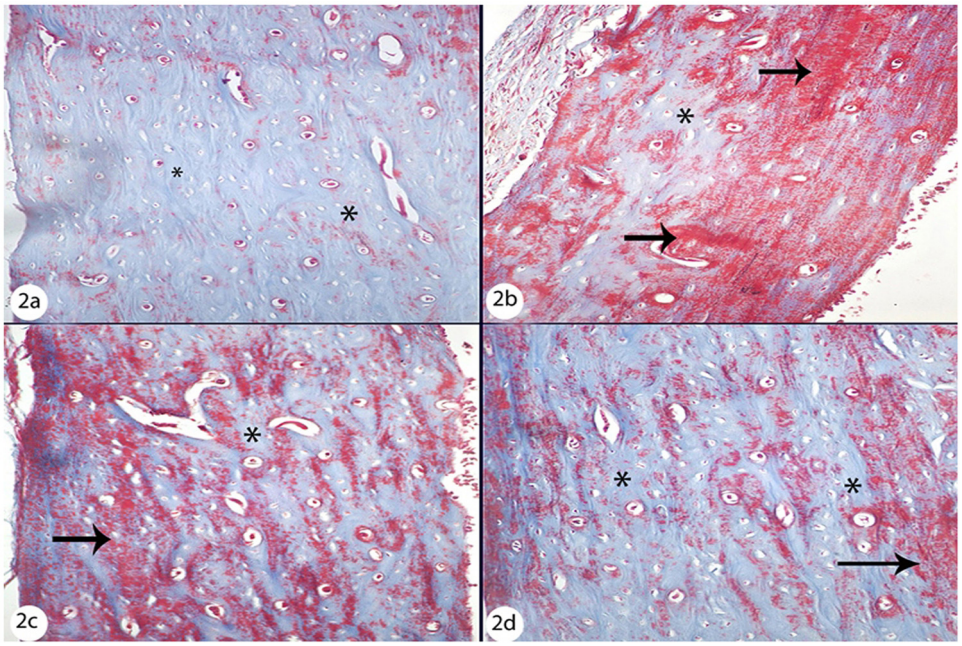

Fig. 2. Masson's trichrome-stained sections. (2a): Control group with lamellar arrangement of abundant blue-stained collagen fibers (*). (2b): Untreated diabetic group with a thin cortex and apparent decrease of the demonstrated collagen (*) (2c): Diabetic + insulin group showing a relative decrease in collagen with a loss of its lamellar arrangement (*). (2d): hesp +insulin treatment appears to results in a normal distribution of collagen fibers (*).The red-stained osteoid (bone organic matrix) (arrows) in all groups is inversely proportional to the amount of collagen. (MT ×400). (For interpretation of the references to colour in text, the reader is referred to the web version of this article.)

number of osteoblasts was detected in the insulin-treated DM group and hesp +insulin treated DM group compared with the untreated DM group. Additionally, a significant difference was detected between the insulin-treated and hesp-treated DM groups ($P < 0.05$).

Measurement of the cortical bone thickness revealed a significant decrease in the untreated group and a significant increase in groups III and IV. However, the cortical bone thickness was significantly decreased in the insulin-treated group compared with the control and hesp +insulin-treated groups.

The mean area percentage of blue-stained collagen fibers in the untreated DM group showed a significant ($P < 0.05$) decrease compared with the control group. However, a significant increase in collagen fibers was observed in the insulin-treated and hesp +insulin -treated DM groups ($P < 0.05$) compared with the untreated DM group and a significant decreased was observed in the insulin-treated group compared with the hesp +insulin -treated group.

3.4. Biochemical results

3.4.1. Serum glucose and bone turn over markers (Table 3)

At the end of the experiment, the injection of STZ increased the mean serum glucose level to 349.6 ± 77.8 mg/dl and decreased the mean serum insulin level compared to the control. These levels were restored to control values in the insulin-treated and hesp +insulin -treated DM groups (Table 3).Similar findings were reported regarding the weekly blood glucose levels in the STZ injected groups (II III, IV) measured by glucometer from tail vein blood (data not shown).

Serum OC and OPN levels in untreated or insulin-treated diabetic groups were significantly decreased compared with the control group. In contrast, the mean serum ALP was significantly increased compared with the control group.

In the hesp +insulin treated group, these parameters were restored to levels that were not significantly different from the control group. Significant increase in the serum osteocalcin and osteopontin levels and a significant decrease in the serum ALP level was detected in hesp +insulin treated group when compared with the untreated DM and insulin-treated DM groups.

3.4.2. TNF-α gene expression levels in rat bone

The mean TNF-α expression level was a significantly increased in the untreated DM group and insulin-treated group compared with the control group ($P \leq 0.001$). Hesp treatment decreased TNF-α expression to a level that was not significantly different from the control group. However, insulin could couldn't maintain normal expression of TNF-α (Fig. 5).

3.4.3. NF-κB gene expression levels in rat bone

The mean NFκB expression level was significantly increased in the untreated DM and insulin-treated group compared with the control group ($P \leq 0.05$). There was also a significant increase in the mean NFκB expression level in the insulin-treated group compared with the hesp +insulin -treated group ($P \leq 0.001$).

Hesp treatment decreased NF-κB expression to a level that was not significantly different from the control group (Fig. 6).

4. Discussion

Osteoporosis is the most important metabolic bone disease in T1DM patients that is thought to be induced in part from oxidative stress (Yamamoto et al., 2009). Hesperidin could be expected to ameliorate diabetes related oxidative stress (Ashafaq et al., 2014). So, this study was conducted to investigate the possible ameliorative effect of hesp supplementation versus insulin alone on osteoporosis caused by T1DM in growing rats. We concerned evaluation of bone in young rats with diabetes just after weaning as they did not reach the peak of bone mass yet and there is lack of concordant results about the impact of T1DM on growing bones that could later affect bone mass (Zhukouskaya et al., 2014).

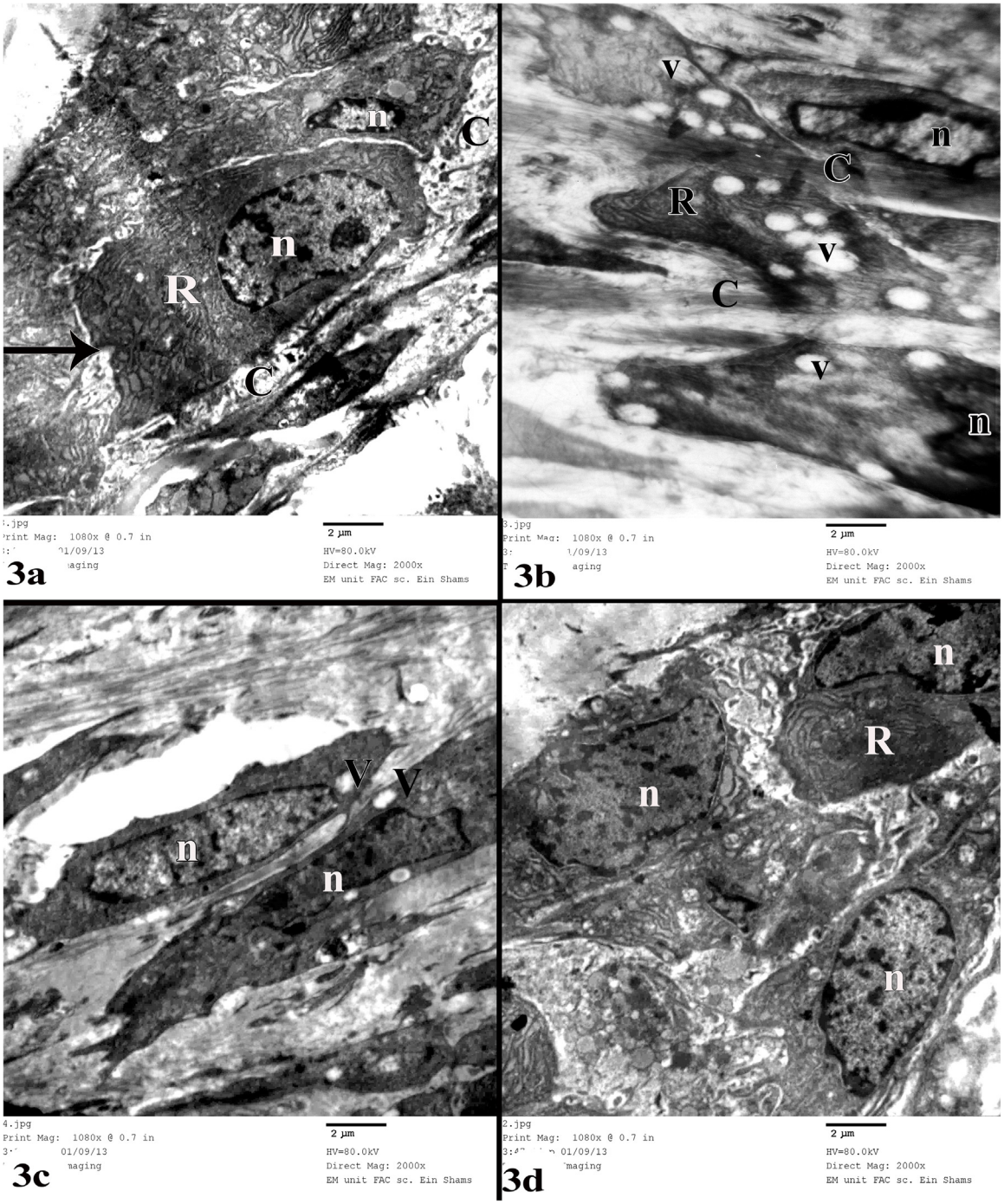

Fig. 3. Ultrathin-sections from shaft of rat femurs. Osteoblasts of the control group (3a) with euchromatic nuclei (n), plenty rER (R) and few cytoplasmic processes (arrows). The cells are surrounded by a layer of osteoid containing collagenous fibrils (C). (3b): osteoblasts of untreated diabetic group are elongated with peripheral condensed heterochromatic nuclei (N), vacuolated cytoplasm (V) and non-prominent rER profile. The abundant translucent matrix contains irregularly arranged collagen fibers (C). (3c): Diabetic + insulin group showing osteoblasts with a vacuolated cytoplasm (V) and non-prominent rER profile (3d): hesp +insulin-treated diabetic group showing active osteoblasts with euchromatic nuclei (n) and prominent rER (R). **(TEM scale bar: 2 µm)**.

Regarding normal rats, there were no effects of hesp administration on bone histological and biochemical markers (data were not shown). Similar findings were detected by Horcajada et al. (2008) and Akiyama et al. (2010).

The present work detected several histological bone changes in untreated rats with diabetes together with hyperglycaemia and hypoinsulinaemia. Manifestations of osteoporosis were observed in the form of degeneration of bone cells with appearance of empty lacunae, multiple erosion cavities, decreased collagen fibers and a rarefied bone matrix. Also, morphometric analysis revealed a reduction in osteoblast numbers and the cortical mass. The previously detected histological affection of long bone was identified by Gordi and Neica (2010) and Marcu et al. (2011) as bone resorption manifestation of osteoporosis.

The previous histological changes were accompanied by a significant decrease in bone turnover markers including serum OPN and OC levels and increased serum ALP in the current study. Similarly, Qing Zhang et al. (2007) and Balci Yuce et al. (2014) detected decreased serum OC and OPN was associated with bone changes including rarefication in diabetes.

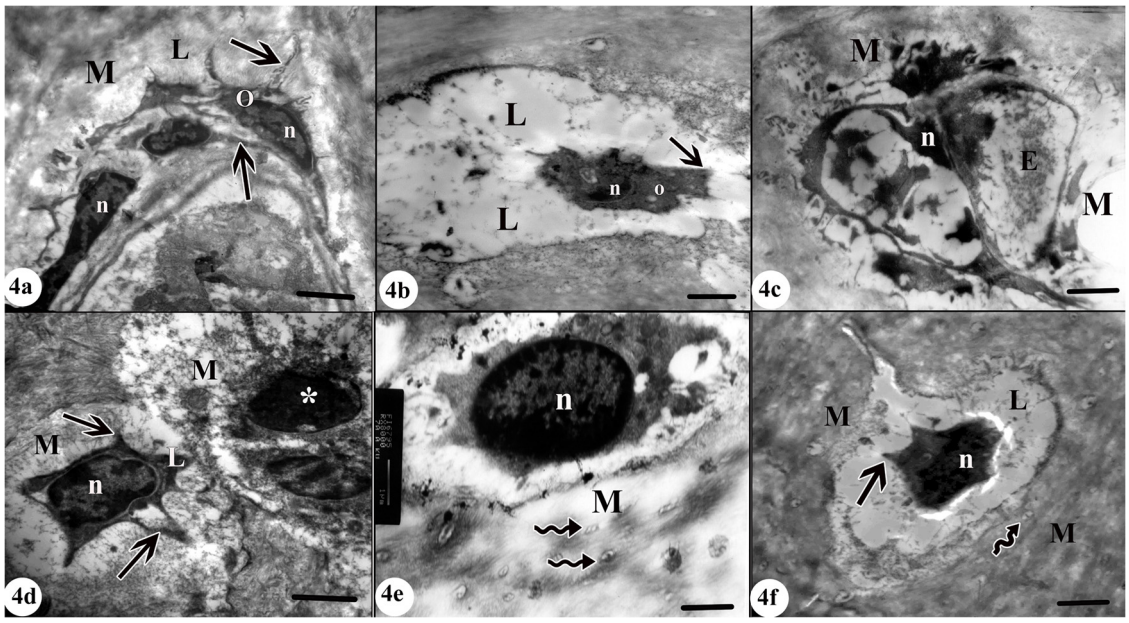

Fig. 4. Ultrathin-sections from the shaft of rat femurs showing: (4a) osteocyte (o) of the control group is stellate shape residing in lacunae (L), containing ovoid nuclei (n), little cytoplasm and cytoplasmic processes (arrows) that extend within the surrounding matrix (M) contain abundant collagen fibrils. (4b & c): Osteocytes (o) of the untreated diabetic group appear shrunken with electron dense nuclei (n) and blunt cellular processes (arrows). Their lacunae (L) appear large. The matrix (M) around the cells is rarefied. (4d & e): Osteocytes of the insulin-treated diabetic group show heterochromatic nuclei and cell processes (arrows). The matrix around the processes is rarefied. In (4e) the cell processes and canaliculi are seen in cross sections (wavy arrows). (4f): Osteocytes of the hesp +insulin-treated group appear as stellate cells trapped in lacunae (L) and surrounded by organized matrix (M). **(TEM scale bar = 2 μm)**.

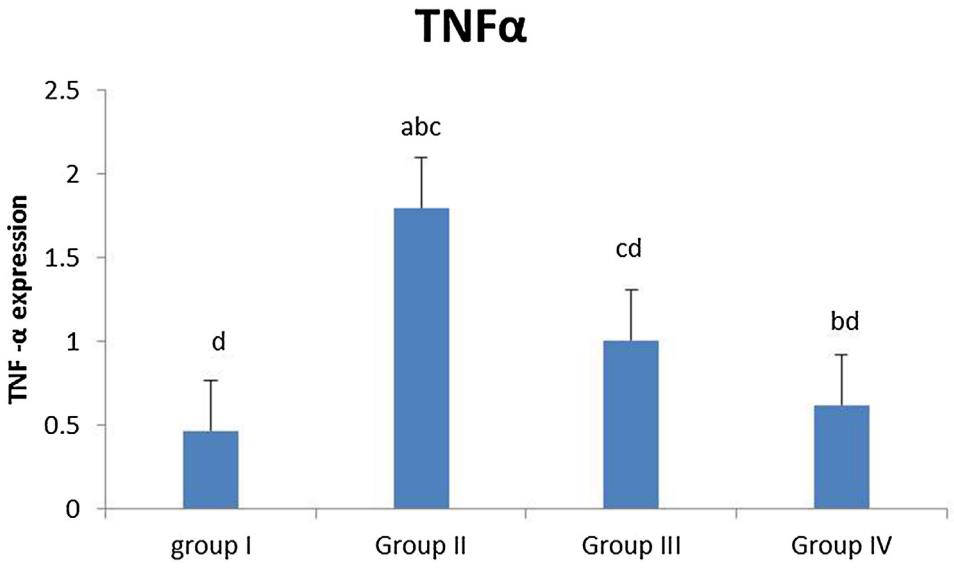

Fig. 5. The mean ± SD of TNF-α gene expression levels in rat bone. a significantly different from group 1, b significantly different from group III, c significantly different from group IV, d significantly different from group II.

Hofbauer et al. (2007) illustrated that absence of the anabolic effects of insulin in patients with type IDM impaired mainly bone formation. Growing evidence suggests that oxidative stress may contribute to the development of diabetic osteopenia that can't be corrected by insulin (Jakus et al., 2012; Erdal et al., 2012; Weitzmann, 2013; Ashafaq et al., 2014).

Osteoblasts are a crucial component of the fracture-healing cascade. Hyperglycemia has variable effects on osteoblasts. Hyperglycemia results in down regulation in genes for cell-signal proteins such as osteocalcin and up regulation of genes for alkaline phosphate (Kalaitzoglou et al., 2016).

Degeneration of osteoblasts and osteocytes was observed in untreated diabetes and not fully corrected with insulin treatment in this work. Osteoblasts were decreased in number and appeared elongated with decreased cytoplasmic area and absence of rER. Most osteocytes appeared with pyknotic nuclei; shrunken cytoplasm and even empty lacuna were commonly observed. Chen et al. (2012) suggested that hyperglycemia affects the development of osteoblasts from mesenchymal stem cells resulting in their death.

Moreover, higher levels of gene expression of the pro-inflammatory mediators TNFα and NF-κB were detected in the untreated and insulin-treated groups in the current study. These

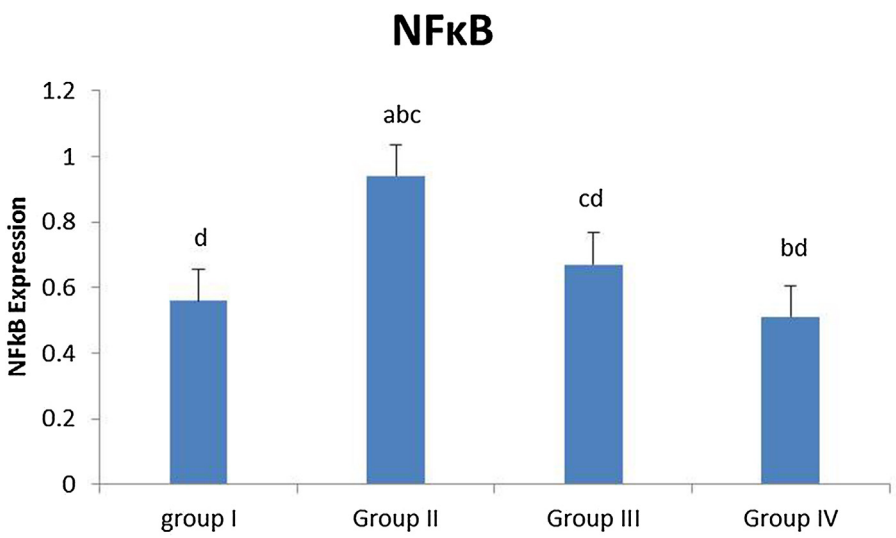

Fig. 6. The mean ± SD of NFκB gene expression levels in rat bone. a significantly different from group I, b significantly different from group III, c significantly different from group IV, d significantly different from group II.

proinflammatory mediators induced the inhibition of insulin receptor phosphorylation thus induced cell apoptosis through prostaglandin, thereby affecting bone structure (Chen et al., 2012). Also, RANKL belongs to the TNF superfamily and is critical for osteoclast formation. So, increased TNF resulted in activated osteoclast and bone destruction (Cohen, 2006). Kalaitzoglou et al. (2016) illustrated that a complex environment was associated with T1DM that impacts osteoblasts, osteocytes, and osteoclasts. Diabetes induced inflammation affects osteoblast number through decrease osteogenesis and increase osteoblast apoptosis and decreases their maturation to osteocytes (Czernik and Fowlkes, 2015).

Poor osteoblast activity and survival resulted in poor bone formation in T1DM was contributed to complex interaction between insulinopenia, pro-inflammatory cytokine production, and alterations in gene expression. The negative impact of T1DM on osteoblasts and osteocytes is well established. Whereas, limited studies available indicate increased osteoclast activity, favoring bone resorption (Kalaitzoglou et al., 2016).

Bone turn over markers (BTMs) can be detected in serum and reflect bone resorption, formation or both. In this work, the compounds chosen to reflect bone turn over were either a part of the matrix (OC, and OPN), collagen as precursors or degradation products of the matrix and enzymes (ALP). Some compounds may have several roles. Alkaline phosphatase is both an enzyme which initializes mineralization, and a marker of osteoblast function. OC is a part of the unmineralized matrix, but also has hormonal properties which would reflect overall bone metabolism, including formation and destruction (Watts, 1999). OPN has been suggested by many studies to be expressed by both osteoclasts and osteoblasts to promote attachments of osteoclasts for bone (Watts, 1999; Shapses et al., 2003; Coleman et al., 2008). Osteoclasts bind to bone matrix *via* integrin receptors in the osteoclast membrane. The main integrin receptor facilitating bone resorption binds to OPN (Ross and Teitelbaum, 2005). So, OPN is bone matrix protein that plays important roles in bone formation, resorption, and remodelling (Wang and Denhardt, 2008).

The present work detected a significant decrease in collagen fibers in either untreated or insulin-treated rats with diabetes. This was explained by Vincent et al. (2002), Jagtap and Patil (2010), Mao et al. (2014). They illustrated that a slight elevation in the blood glucose level leads to non-enzymatic glycosylation of the organic matrix due to the formation of advanced glycation end products that degenerate collagen fibers.

Decreased amount of collagenous matrix together with decreased serum levels of OC and OP and increase ALP were a marker of decreased bone turnover resulting in higher fracture incidence with T1DM (Neumann et al., 2016; Starup-Linde and Vestergaard, 2016).

Although treatment with insulin in this work restored the serum glucose and insulin levels towards normal, the histological structure of the bone still affected. Serum ALP was increased and serum OPN was decreased. Conversely, improvement of the histological and biochemical data from bones toward normal was detected following hesp supplementation. Erdal et al. (2012) found that insulin treatment was not sufficient for bone health because an elevated fracture risk was still present in T1DM patients. Additionally, Zhukouskaya et al. (2014) reported that other factors besides hyperglycaemia such as autoimmune inflammation and an IGF-1 deficit could interfere with bone health and were not corrected by insulin.

Hesperidin +insulin supplementation of rats with diabetes prevents osteoporosis that proved in our study by increase in cortical bone thickness, osteoblast number and collagen expression by masson's trichrome. Also, BTM including OC, OPN and ALP were returned to normal values together with normalization of the serum level of glucose and insulin.

Similar to our results, other studies reported a decrease in inflammatory mediator production after tissue exposure to hesp (Comalada et al., 2006; Rathee et al., 2009).

The hypoglycemic effect of hesp was reported previously in type 1 DM (Akiyama et al., 2010) and type 2 DM (Jung et al., 2006). They illustrated that hesp increases the mRNA level of glucose catabolism key enzyme Glucokinase (GK), and decrease gluconeogenic enzyme level (G6Pase). Also, Osteocalcin itself that was increased after hesp treatment is reported to increases insulin secretion and sensitivity (Lee et al., 2007; Ferron et al., 2008; Confavreux et al., 2009).

Those findings indicated the effect of hesp as antidiabetic agent by decreasing blood glucose and increasing insulin level. Although, a protective role for hesp in diabetes has been established in previous studies by its antioxidant property in many tissues including heart, liver and kidney (Gumieniczek, 2003; Wilmsen et al., 2005; Akiyama et al., 2010), other studies reported an antidiabetic effect of hesperidin (Akiyama et al., 2010; Kim et al.,

2013). These studies illustrated that hesp accentuated insulin secretion through the attenuation of B-cell damage and a decrease in glucose absorption from the intestine, thereby reducing the blood glucose levels.

Trzeciakiewicz et al. (2010) suggested that hesp regulated osteoblast differentiation through bone morphogenic protein signalling and might influence the mineralization process by modulating OPN and OC expression. These phenomena increase the proliferation and activity of osteoblasts and inhibit the generation and activity of osteoclasts. Moreover, matrix organization and collagen fiber proliferation were reported to be affected by hesp (Choi and Kim, 2008; Partridge et al., 2008; Trzeciakiewicz et al., 2010; Kim et al., 2013).

Based on these results, it is clear that T1DM induces many structural changes in bone at growing age, giving the picture of osteoporosis, affects bone turnover markers and increases the gene expression of proinflammatory mediators. These changes were ameliorated by hesp which is a cheap, readily available natural plant extract. Therefore, hesperidin could be used in the future as a curative measure against diabetic osteoporosis. Further studies are strongly recommended to understand the underlying mechanism of protective action of hesp. Also, achieve a combination therapy for the treatment and prevention of diabetic osteoporosis need further studies.

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No conflict of interest to disclose.

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