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Research Article

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Evaluation Effect of Pearl Millet Aqueous Extract and Simvastatin on Mineral Composition and Histology of Lumbar Vertebrae in Wistar Rats

Ali Kaddafi[1,2*], Bouchra Meddah[2], Omar Elblidi[1], Sana Chala[3], Yahya Charrah[2], Omar Chokairi[1] and Malika Barkiyou[1]

[1]Laboratory of Histology, Embryology and Cytogenetics, Mohammed V University, Rabat, Morocco
[2]Laboratory of Pharmacology and Toxicology, Mohammed V University, Rabat, Morocco
[3]Department of Endodontics and Restorative Dentistry, Laboratory of Clinical and Epidemiological Research, Mohammed V University, Morocco

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ABSTRACT

A high-fat diet has been reported to induce negative effect on rats' bone mineral composition, microstructure and architecture. The aim of the present study is to assess the effect of aqueous extract of Pennisetum glaucum (AEPG) compared to simvastatin (SIM) on the lumbar vertebrae and Kidney for female rats fed on a cafeteria diet. Thirty seven-weeks old female rats (50-60 g) were weighted and divided in two groups: a control group (n=10), fed on a standard diet (SD) and an experimental group (n=20) receiving cafeteria diet (CD)-SD for 22 weeks to induce obesity. The experimental group was divided in three groups: CD group (n=6) continued receiving CD+SD, CD-AEPG group (n=7) received CD+SD+AEPG (250 mg/kg of body weight (b.w.)), CD-SIM group (n=7) received CD+SD+SIM (10 mg/kg b.w.). All lumbar vertebrae and kidneys were removed and weighted. The lumber (VL_{1-2}) and kidney biopsies were taken for histopathological evaluation. The lumbar (VL_3) is used for scanning electron microscopy and lumbar (VL_4) used for analysis of mineral composition. We noted that the AEPG supplementation induced significant decrease of kidney weight compared to the control group. The lumbar weight has increased in CD, CD-AEPG and CD-SIM groups than control group ($p=0.04$, $p=0.05$, $p=0.05$ respectively). Our results reported that calcium and phosphor content decreased in CD group compared to the control. We noted a significant improvement of bone lesions under AEPG and SIM treatment. To conclude, AEPG and simvastatin administration decreased the bone alteration of the rats fed on a cafeteria diet. The AEPG possesses antiosteopenia proprieties.

Keywords: *Pennisetum glaucum*; Simvastatin; Obese rat; Lumbar vertebra; Kidney

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INTRODUCTION

Epidemiologic and clinical studies show that a high level of fat mass might be a risk factor for osteoporosis and fragility fractures [1]. The relationship between obesity and high-fat diet consumption is well demonstrated. Zhou and al. reported that diet-induced obesity is strongly associated with severe destruction of bone architecture and reduces potential for bone regeneration [2]. Nutrition, including high-fat diet-induced obesity and insufficient caloric intake, may contribute to the likelihood of bone disease [3] with increase bone resorption and impair bone microarchitecture [4]. When fed a high-fat diet for two years, rats had a significantly smaller average cross-sectional area of the sixth lumbar vertebra [5] and showed numerous alterations of bone structure and mechanical proprieties. Previous studies have demonstrated that kidney dysfunction is implicated in the bone pathology. Osteomalacia is accelerated by inadequate renal phosphorus wasting and decreased uptake of active vitamin D [6].

Anti-osteoporosis, anti-osteopenia drugs such as calcitonin, bisphosphonate, and estrogen have been used and their bone protection mechanisms are based on the inhibition osteoclastic bone resorption [7]. Statins regroup the most hydroxymethylglutaryl-coenzyme A reductase inhibitors, which are commonly prescribed in lipid-modifying

therapies [8]. Mundy et al. reported that the statins, drugs widely used for lowering serum cholesterol and enhancing new bone formation *in vitro* of rodents [9]. Simvastatin (SIM) has been used to treat or prevent many disorders affecting the bone tissues and stimulate bone regeneration [10]. Recent study suggested that simvastatin is a potent osteoinductive factor that can increase action of filaments organization and cell rigidity in mesenchymal stem cells [11]. Synthetic drugs have several side effects, causing diverse complications for many patients. Numerous people use folk medicine to treat diseases and have fewer undesirable side effects. Pearl millet (poaceae) has been used in Moroccan folk medicine to treat bone fracture, trauma and bone disease, but this practice is not demonstrated scientifically. In our previous studies, we have found that the aqueous extract of *Pennisetum glaucum* (AEPG) has anti-inflammatory and antioxidant effects [12]. The aim of this present study is to assess the effect of *Pennisetum glaucum* aqueous extract on the lumbar vertebrae and kidney in female rats fed on cafeteria diet versus simvastatin treatment.

MATERIALS AND METHODS

Animals

The 6-week-old Wistar rats (55 – 70 g) were used for these experiments respecting all the Rabat Medical and Pharmacy School IRB. They were housed three per plastic cages (42 × 26 × 18 cm) in a room at 25°C controlled temperature, humidity relative of 55% and having in 12 h light/ 12 h dark cycle. All animals were provided with water and standard diet ad libitum and allowed to acclimate for one week. All procedures performed throughout the experiment conformed to the guidelines of Council instructions about the protection of living animal used in scientific investigations [13].

Cafeteria Diet

The cafeteria diet adopted, providing modified versions of Harris (1993) high fat cafeteria diet [14]. It consisted of 3 variants: (i) condensed milk + bread + peanuts + pellet chow (4:1:4:1), (ii) chocolate + biscuits + dried coconut + pellet chow (3:2:4:1), and (iii) cheese + boiled potatoes + beef tallow + pellet chow (4:2:4:1). The different variants were presented on alternate days throughout the treatment period [15].

Sample Preparation Plant

Whole grains of pearl millet (*Pennisetum glaucum* L.) were obtained from Rabat herbalists (Morocco). The grains identified by the botanic department of the National Scientific Institute, were dried at room temperature and grounded to obtain a fine powder using an electric blender. For the aqueous extract of *Pennisetum glaucum* (AEPG), 1 kg of fine powder was mixed with distilled water (3 volumes) in a glass jar and left for 2 days at room temperature in an orbital digital agitator (Rotatest 560VIT. 15-300 T/MIN). The solvent was filtered through Whatman pleated filter paper number 3. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator [16] and stored at – 20°C until further used.

Experimental Design

Thirty seven-weeks old female rat (50-60 g) were randomly assigned and divided in two groups: control group (n=10), feed on a standard diet (SD) and experimental group (n=20) receiving cafeteria diet (CD)-SD for 22 weeks to induce obesity. All the experiments were conducted between 10:00 and 12:00 h, as follows:

- A untreated control (n=10), feeding standard diet(SD)
- B group (n=20), receiving cafeteria diet CD-SD

Experimental group was divided in three groups: control for CD group (n=6) continued receiving CD+SD exclusively, CD-AEPG group (n=7) received SD+CD+AEPG (250 mg/kg of body weight (b.w.)) preparation, CD-SIM group (n=7) received SD+CD+SIM (10 mg/kg b.w.).

The AEPG was suspended in distilled water and administered orally for 5 day/week at a constant volume (250 mg/kg b.w.) for 4 weeks [16]. At the end, rats were weighed and then anesthetized with an inhalation of diethyl ether. Briefly, all lumbar vertebrae and kidney were removed and cleaned of all soft tissue, and immediately weighted by electronic balance to determine the wet bone weight (WW) and their length and width were measured with caliper (Brzóska MM 2005). The samples were fixed in 10% neutral buffered formalin. The lumbar (VL_1) used for analysis is of mineral composition by inductively coupled plasma (ICP). The lumbar vertebrae (VL_{2-3}) and kidney biopsies were taken for pathological evaluation. The lumbar (VL_4) is used for scanning electron microscopy.

Analysis of Bone Mineral Composition

The lumbar vertebra (VL_1) was chosen for chemical composition analysis. The lumbar vertebra (VL_1) was cleaned of muscular and conjunctive tissues and weighted to determine the wet weight (WW), and placed in a 70°C incubator (Schutzart DIN 40050 IP-20, Germany) for 48 hours. The dried weight (DW) was evaluated to determine the percentage of water content (% water= [(WW-DW)/WW] ×100). After recording the dry weights, the bones were digested by placing them in a solution of 5 mL concentrated nitric acid (NHO_3), 3 mL 70% perchloric acid ($HClO_4$) and 2 mL 35% hydrogen peroxide and microwaving at 180 PSI and 180°C [17]. Mineral contents were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon Ultima 2, French).

Preparation of Specimens for Histology Evaluation

Lumbar vertebrae (VL_{2-3}) were fixed in 5% neutral-buffered formalin for assess bone histology. After decalcification in 5% nitric acid, the samples were dehydrated by graded series of alcohols from 50%-100%. The specimens were cleared and impregnated with toluene. Following dehydratation, the kidney and bone samples were embedded in paraffin and 5 µm thick slices were taken from the length, and stained with Masson's Trichrome. The slides were observed by light microscope [18].

Scanning Electron Microscopy Analysis

The Bone structure was evaluated by scanning electronic microscopy. After fixation and undercalcified bone samples were dehydrated through a graded series (70%, 90%, 95% and 100%) of alcohol [19]. Before microscopy evaluation, thin layer of carbon was covered on the surface of samples, and then evaluated in environment SEM operating (Philips XL Series, FEI Quanta 200) at 30 kV.

Statistical Analysis

Data was presented as the mean ± SD for ten rats per group (n=6). Multiple comparisons were analyzed between groups using one way analysis of variance (ANOVA) and Bonferroni's multiple comparisons test. Significance was established at $p<0.05$ comparing all treatment groups to control. Data analyses were performed using the statistical program Graph Pade Prism version 6.

RESULTS AND DISCUSSION

Body Weight and Macroscopic Analysis

At the beginning, no significant differences were observed in the animals. Figure 1 shows body weight during the diet period evolution. The body weight changed significantly during the experimentation in CD, CD-AEPG and CD-SIM groups compared to the control group (Figure 1A). During the treatment period, we reported no significant differences observed in the CD-AEPG group compared to the CD-SIM group (Figure 1B). We also noted that body mass significantly decreased in AEPG and SIM compared to the CD group [20].

In this study, we also measured Kidney and lumbar vertebrae weight (Figures 1C and 1D). We observed that the kidney and lumbar mass in the CD group was greater than control group. The AEPG treatment didn't significantly decrease the lumbar vertebrae and kidney weight compared with the CD group. Animals treated with SIM showed no significant decrease compared to CD groups. AEPG shows a better result than Simvastatin in weight reducing compared to the CD group especially in the kidney.

Macroscopic data are measured after animal sacrifice. The value of lumbar length statistically increased in AEPG and SIM groups compared to the control group ($p<0.05$ and $p<0.01$ respectively) and CD group. Our results revealed that both lumbar width and water showed no significant differences between groups (Table 1).

Mineral Composition

Mineral analyses showed that calcium and phosphorus content significantly decreased in groups fed on cafeteria diet compared to the CTL group (Table 2). We noted no significant magnesium differences between CD treated and untreated groups. Furthermore, AEPG administration significantly increased zinc content compared to the CD group ($p<0.05$). On the other hand, it is abruptly increased under Simvastatin. Overall, the mineral content was lower in the experimental groups than in the control groups.

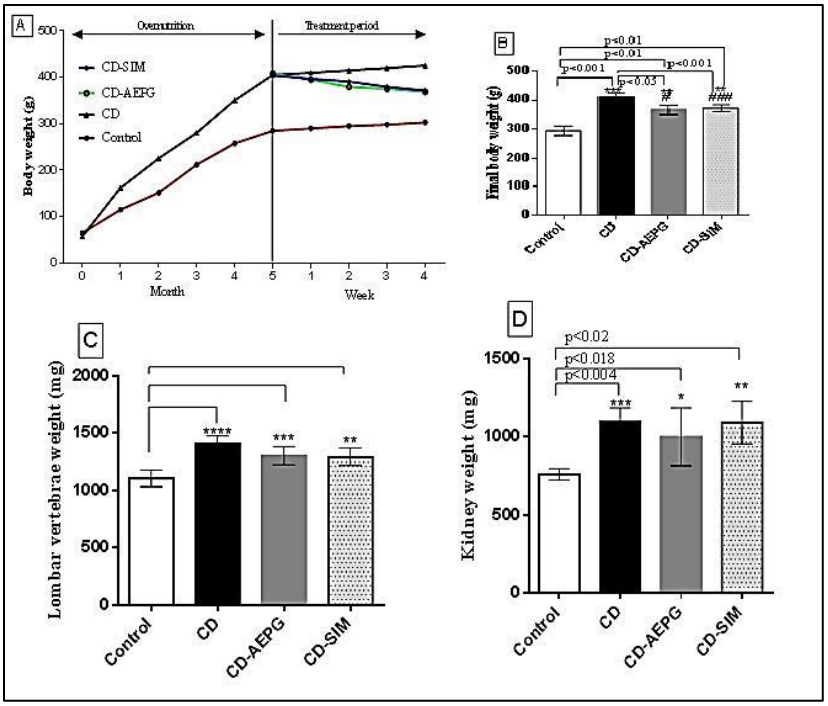

Figure 1: Effect of AEPG and SIM therapy on body mass, lumber weight and kidney weight in rat fed a chow diet, cafeteria diet during 152 days of experimentation. All data are mean ± SEM

Table 1: Effect of AEPG versus simvastatin on macroscopic parameters of lumbar vertebrae in rats fed on cafeteria diet

Groups	Lumbar length (mm)	Lumbar width (mm)	Lumbar water (%)
CTL	34.080 ± 2.154	6.667 ± 0.516	0.518 ± 0.012
CD	36.670 ± 1.211	6.667 ± 0.516	0.526 ± 0.022
CD-AEPG	37.670 ± 3.141[a]	6.500 ± 0.547	0.508 ± 0.020
CD-SIM	38.580 ± 0.801[a]	6.417 ± 0.491	0.491 ± 0.026

Data are expressed as mean ± SEM. CTL: control group, CD group: cafeteria diet, CD-AEPG group: cafeteria diet + *Pennisetum glaucum*, CD-SST: cafeteria diet + simvastatin. [a]p<0.05, [a]p<0.01vsCD-AEPG group and CD-SIM group respectively. [a]p<0.05 vs control group

Table 2: Effect of AEPG versus Simvastatin on lumber vertebra mineral composition in rats fed on cafeteria diet

Parameters	CTL	CD	CD-AEPG	CD-SIM
Ca (%)	18.080 ± 0.262	15.290 ± 0.917[a]	16.100 ± 3.126	16.810 ± 3.006
Mg (%)	0.541 ± 0.317	0.200 ± 0.010	0.211 ± 0.017	0.260 ± 0.101
P (%)	18.670 ± 0.646	13.500 ± 0.288[a]	13.740 ± 1.157[a]	14.880 ± 4.179
Zn (%)	0.042 ± 0.005	0.027 ± 0.000	0.0303 ± 0.001[b]	0.122 ± 0.159

Data are expressed as mean ± SD. CTL: Control group, CD group: cafeteria diet, CD-AEPG group: cafeteria + *Pennisetum glaucum*, CD-SIM group: cafeteria diet + simvastatin. Ca: calcium; Mg: magnesium; P: phosphor; Zn: zinc. [a]p<0.01vs CD (Ca); [a]p<0.0001, [a]p<0.01vs CD group, CD-AEPG group (P) respectively. [b]p<0.001 vs CD-AEPG (Zn); [a]p<0.05 vs control group, [b]p<0.05 vs CD group

At the end of the 5-week follow-up, the cafeteria diet induced a significantly loss lumbar vertebra mineral content for female rats. Overall, no significant differences were observed between groups fed on cafeteria diet treated and untreated. Several studies reported that high fat produced deleterious effects on the absorption of dietary calcium and consequently an adverse effect on bone mineralization in growing animals [21]. This could explain the results obtained in our study. However, we noted that AEPG and SIM treatment generated less mineral content for rats fed cafeteria diet. So, we can conclude that AEPG and SIM treatment seem improve mineral content induced by CAF diet compared to the control group.

Kidney Histology Evaluation

The link between obesity and renal disease is unclear, and there is no consensus as to whether obese individuals are at increased risk for kidney disease [22].

But according to Karen [23] emerging evidence indicates that obesity, even in the absence of diabetes, contributes significantly to the development and progression of chronic kidney disease (CKD). Glomerular hyperfiltration and hypertrophy in response to the increased metabolic needs of obesity are postulated to lead to the development of glomerulosclerosis.

Obese people show an increase in the glomerular size and a variable degree of mesangial expansion. Our experiment shows normal glomeruli structure in control group (Figure 2(a1)) and glomerular hypertrophy and fibrous in the CD group which comfort Karen [23] observations. As regards the accumulation of fat vacuoles which we observed in kidney sections from 7months old female rats fed on cafeteria diet for 6 months (Figure 2(b1)), we can say that we are in the same line with Mohamed WS et al. [24] who indicate that the cells of the lining epithelium of proximal convoluted tubules revealed many vacuoles scattered in the rarefied cytoplasm. Taking account of all these data we can conclude that cafeteria diet induced kidney damage and inflammation.

In our Work, treatment with AEPG caused a significant improvement and prevention of fatty deposition in nephron tubules, and reduction of kidney hypertrophy (Figure 2(c1)). Improvement of kidney damage which is materialized by decrease of fat vacuoles in nephron and glomerular size were seen also with simvastatin (Figure 2(d1)). Our results concerning simvastatin are in concordance with Hiroaki Oda et al. [25]. Statins and other lipid-lowering agents may influence important intracellular pathways [25] involved in the inflammatory and fibrogenic responses observed in many forms of progressive renal injury.

Thereby, since there is no work that deals with the therapeutic action of AEPG for kidney damage, we can conclude to the similarity of its actions with those of simvastatin.

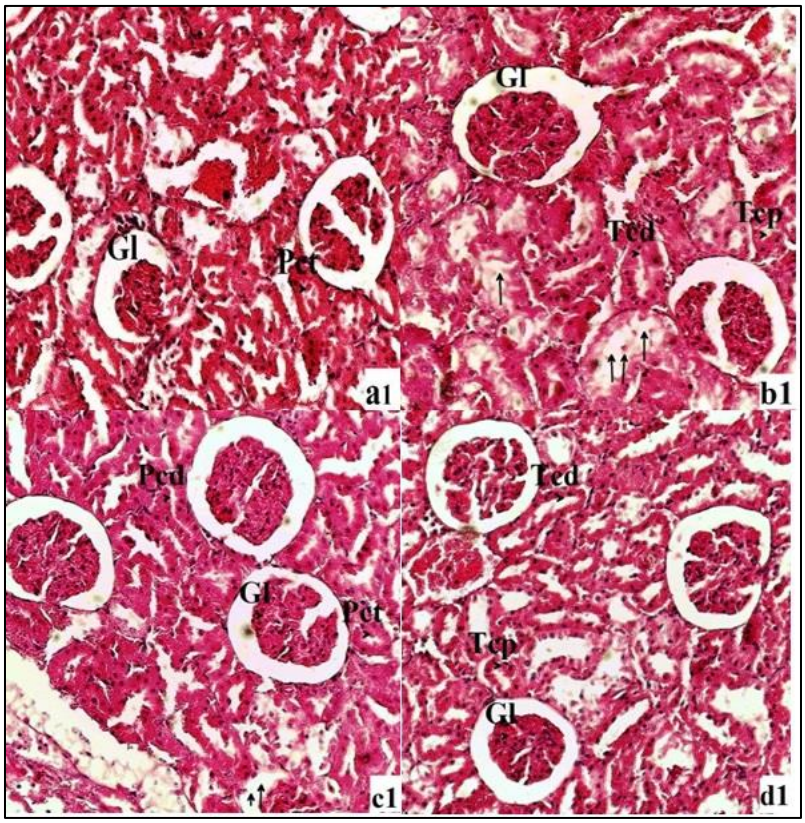

Figure 2: Effect of AEPG and Simvastatin on kidney histopathological in rats feeding cafeteria diet. The samples is staining in hematoxylin-eosine (200 × magnification). (a1) control group, (b1) CD group, (c1) CD-AEPG group, and (d1) CD-SIM group. Gl: glomerul; Pct: proximal convoluted tubule, Dct: distal convoluted tubule. Black arrows denote fat vacuoles

Bone Histological Evaluation

Some investigations demonstrated that short-term and extended high-diet feeding male mice increased bone resorption and significantly impaired bone density and bone microarchitecture [4].

As seen above, in obese mice, our experiment histological evaluation in rats feed on cafeteria diet showed deteriorated trabecular architecture, increased bone resorption and higher adiposity which were materialized by increased fat vacuoles. Also the bone morrow decreased compared to the control group (Figure 3A). In CD group,

we observed in addition, that (Figure 3B) cortical porosity and the trabecular thickness bone are affected. We noted additionally, irregular bone shape and more megalocytes.

The treatment with AEPG at dose of 250 mg/kg showed a significant positive improvement on bone structure (Figure 3C), the fall of number of fat microvacuoles and improved the bone marrow differentiation compared to the CD group. We also remarked that AEPG administration attenuated the expression of osteoclast cells.

Similar observations like those with AEPG were noted in rats treated with simvastatin (Figure 3D) at the dose of 10 mg/kg.

In our study, we observed that simvastatin and AEPG boosted not only enhanced trabecular bone, but also increased osteocytes lacuna in lumbar vertebra significantly. Garrett et al. reported that statins treatment increase bone formation and bone mass in rodents, which suggests a potential benefic action in patients with established osteoporosis [26]. On the fundamental plane Dai et al. indicated that the anti-osteoporosis activity of simvastatin may be due to the promotion of proliferation and differentiation of osteoblasts [27]. For Takeno et al. simvastatin has anti-apoptotic effect on osteoblasts and osteocytes by reducing oxidative stress [28].

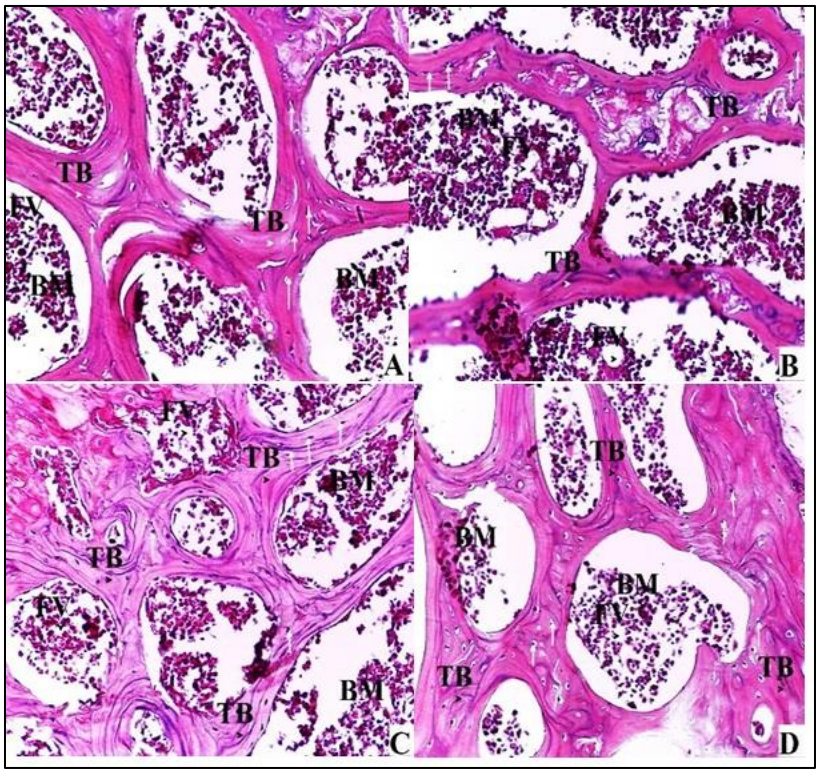

Figure 3: Effect of AEPG and SIM on lumbar vertebra (LV_3) histopathology. Hemalun-eosin staining architecture images of lumbar vertebrae (200 × magnification). (A) Control group, (B) CD group, (C) CD-AEPG group, (D) CD-SIM group in rat. TB: trabecular bone; BM: bone marrow; FV: fat vacuoles; white arrows denote the lacuna osteocytes

In the present study, we used Masson trichrome to evaluate bone architecture and collagen fibrous expression. This coloration technique showed that collagens fibrous are less developed in CD group (Figures 4A and 4B). The administration of AEPG was essential to correct collagen fibrous depletion (Figure 4C), and enabled a modest development, but is still significantly interesting. As shown in Figure 4D, the effects of simvastatin on bone formation were sensibly similar to AEPG administration. Concerning simvastatin, recent study showed that this drug increased the mechanical parameters such as maximum load, stiffness, and improved microarchitecture [27]. In both treatment by AEPG and SIM, megalocytes were less expressed compared to the CD group.

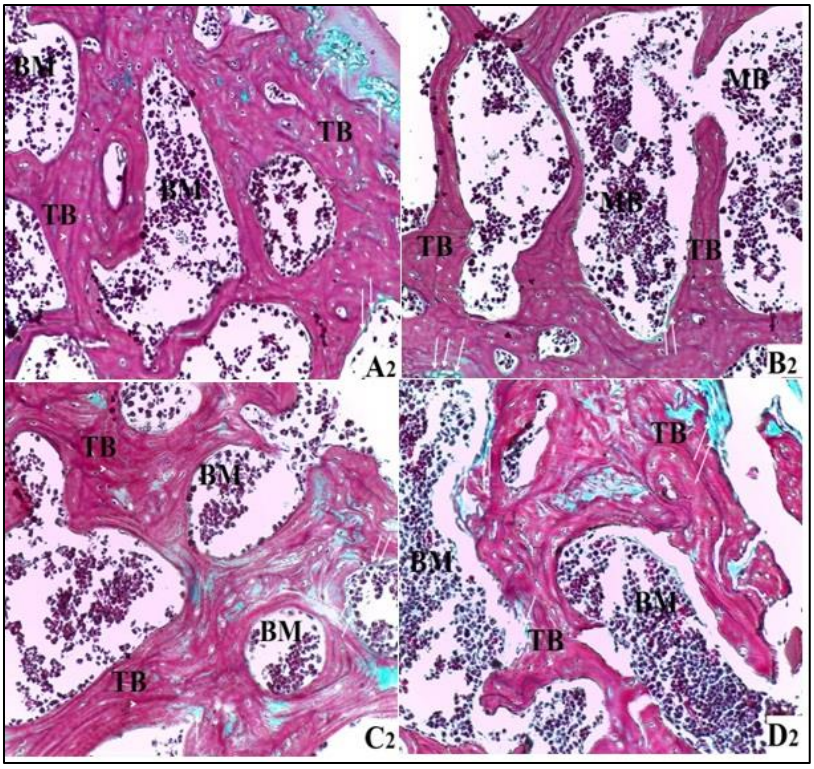

Figure 4: Effect of AEPG and SIM on lumbar vertebra (LV_3) histopathology. Masson trichrome staining micrographs of lumbar vertebra (200 × magnification). (A2) Control group, (B2) CD group, (C2) CD-AEPG group, (D2) CD-SIM group in rat. TB: trabecular bone; BM: bone marrow; white arrows denote the collagen fibrous

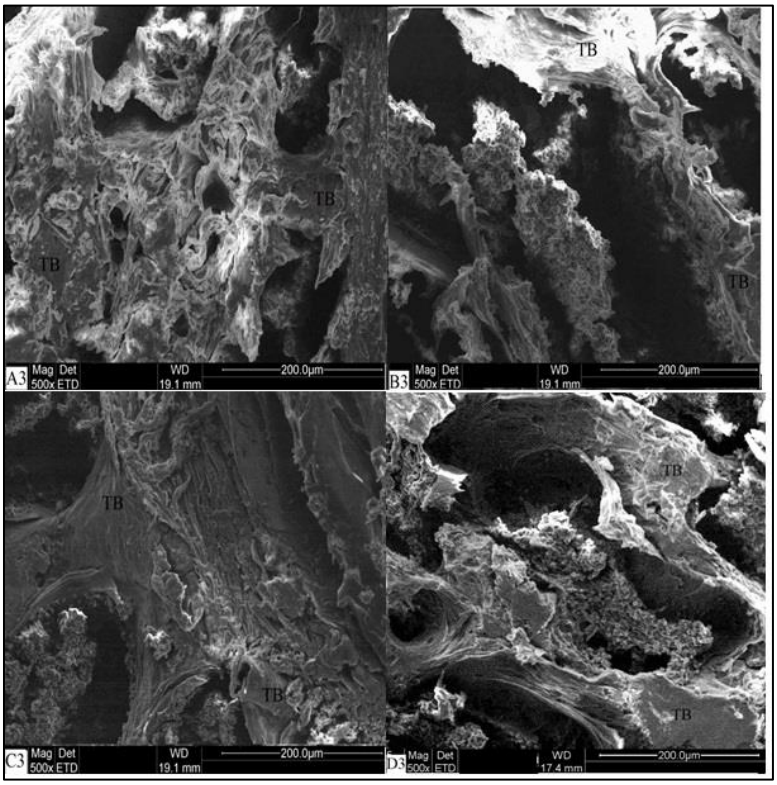

Figure 5: Histologic study of destruction lumbar at 4 weeks after AEPG and simvastatin treatment (500× magnification): (A3) CTL group; (B3) CD group; (C3) CD+AEPG group; (D3) CD-SIM group. TB: trabecular bone

Bone Microarchitecture

The scanning electronic microscopy has been used to explore microstructure for the lumbar vertebra (LV_4). We noted that the trabecular bone microarchitecture was affected in CD group (Figure 5). Our results showed that CD decreased the bone formation and increased bone volume and bone resorption compared to the control group.

We share precisely the same results as for whom short-term and extended high-diet feeding male mice increased bone resorption and significantly impaired bone density and alteration bone microarchitecture [4]. In our experiment simvastatin and AEPG treatment increased microarchitecture proprieties of trabecular bone and attenuated the cafeteria diet effect. Trabecular bone quality was improved with these two treatments.

Many studies have formally established the beneficial role of SIM on bone damage caused by obesity [3,26]. Our work on rats fed cafeteria diet has also shown that AEPG brings a real benefit in the improvement of bone alterations, span architecture and depletion of collagen fibers.

CONCLUSION

Research on the benefit of simvastatin in renal and bone damage subsequent to obesity are numerous and well-founded. The treatment with AEPG in rats, which we induced obesity showed similar effects as simvastatin drug concerning bone lumbar vertebrae and kidney involvements. The main interest of the AEPG treatment is its low cost, availability and its natural origin. All these characters make it a very interesting plant in the treatment and prevention of bone alteration secondary to obesity. The next step is to determine accurately the therapeutic doses and adverse effects of this natural therapy.

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