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Original Research Article

Effect of Kelulut honey supplementation on bone health in male rats on high-carbohydrate high-fat diet

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Abstract

Purpose: *To determine the effects of Kelulut honey (KH) on the bone health of rats with metabolic syndrome.*
Methods: *Male Wistar rats were randomised into normal control and metabolic syndrome s fed with a diet enriched with carbohydrate and fat. The rats in the metabolic syndrome arm were further assigned into the negative control group and honey group supplemented orally with Kelulut honey (1g/kg) daily for eight weeks. After the rats were sacrificed, the trabecular and cortical micro-architecture of the harvested femur was analysed using X-ray micro-computed tomography, while histomorphometric method was used to determine bone cell indices. Femoral biomechanical properties were analysed using a universal mechanical tester.*
Results: *Total cross-sectional area, osteoid surface and volume, displacement and strain reduced significantly, while eroded surface increased significantly in the rats with metabolic syndrome than the normal rats ($p < 0.05$). The honey group showed a significant reduction in osteoclast surface than the normal healthy control ($p < 0.05$). Other skeletal parameters did not show a significant intergroup difference.*
Conclusion: *Metabolic syndrome is harmful to bone health and honey has limited effects in reversing these negative effects.*

Keywords: *Calcium, metabolic syndrome, Osteopenia, Osteoporosis, Bone health*

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INTRODUCTION

Metabolic syndrome (MetS), which consists of a group of metabolic conditions, escalates individual risk for cardiovascular diseases and diabetes. Hyperglycaemia (elevated triglycerides and reduced high-density lipoprotein cholesterol), central obesity, dyslipidaemia, hypertension and are among the components of MetS [1]. A systematic review in 2017 estimated that the

adult population affected by MetS globally ranges from 20 to 25 % [2]. MetS may invoke metabolic complications on skeletal health through chronic inflammation and oxidative stress, increase osteoclast formation and bone resorption activities, promote differentiation of mesenchymal stem cells to adipocytes instead of osteogenic progenitors, and increase calcium excretion [3]. Although the overall influence of MetS on the skeletal system is debatable, many studies feeding high-carbohydrate high-fat (HCHF) diet in rats have suggested that it harms bone health through deterioration of bone micro-architecture and mechanical strength [4,5].

Honey is a healthy food made by the Apis genus of bumblebees. It contains various micronutrients, enzymes, polyphenols, vitamins and trace elements [6]. Kelulut honey (KH), produced by stingless bumblebees, is found in Malaysia. It contains phenolic acids, such as 4-hydroxyphenyl acetic acid, caffeic acid derivatives, coumaric acid, and gluconic acid [7]. Ramli et al established that KH reduced body fat percentage by causing adipocyte hypertrophy, blood pressure and serum triglyceride level in rats fed with HCHF diet [7].

Many preclinical studies have reported the skeletal-beneficial effects of honey. Tualang honey, a type of tropical honey in Malaysia, was found to prevent deterioration of skeletal micro-architecture in ovariectomised rats [8,9]. The bone-protective properties of honey are attributed to the presence of antioxidants and polyphenols [10]. Polyphenols enhance bone formation by promoting the differentiation of mesenchymal stem cells to osteoblast progenitors and influencing signal transduction pathways in bone cells, such as Wnt, bone morphogenic protein, forkhead box protein O3, runt-related transcription factor 2 and osterix, that promote bone formation [11,12]. Quercetin and kaempferol, commonly found in honey, enhance osteoclast apoptosis, thereby inhibiting bone loss [13]. Kaempferol also prevented castration-induced bone resorption by promoting osteoblast function [14]. However, there are limited studies on the influence of KH on skeletal health currently.

The current study examined the actions of KH on skeletal health defined by femoral micro-architecture, biomechanical strength, and histomorphometry in rats with MetS induced by the HCHF diet. KH supplementation was hypothesised to reverse the adverse effects of MetS on the skeletal system. The results of the phytochemical analysis of KH and the metabolic outcomes of MetS-supplemented rats have been published elsewhere [7], so they were not included in this paper.

EXPERIMENTAL

Preparation of KH

A local honeybee farm in Gombak, Selangor, Malaysia, provided unprocessed KH obtained from *Heterotrigona itama* (stingless honey bees). The honey was kept at 4°C in a glass jar before use. KH was mixed with purified; water (ratio of 1:1) for oral administration.

HCHF preparation

The composition of HCHF diet given to the rats is presented in Table 1. Fructose powder (Merck, New Jersey, USA) (25 mg/100 mL) was added into the drinking water of the HCHF group. Access of rats to food and water was not limited.

Table 1: Composition of HCHF diet

Ingredient	Manufacturer	Weight
D-(-)-Fructose	Merck, Darmstadt, USA	175 g
Emprove®		
Sweetened condensed milk	Fraser & Neave Holdings Bhd., Kuala Lumpur, Malaysia	395 g
Enrico's Pure Ghee	Raviraj Sdn. Bhd., Penang, Malaysia	200 g
Hubble, Mendel and Wakeman salt mixture	MP Biomedicals, California, USA	25 g
Powdered rat chow	Gold Coin Feedmills (M) Sdn. Bhd., Selangor, Malaysia	155 g
Tap water	-	50 g/kg diet

Animals

The Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia, provided the 24 male Wistar rats (250 – 300 g; three months old) used in this study. Before being used, the rats were acclimatised to the housing facilities for two weeks. The rats dwelt individually in ventilated plastic cages at the Animal Laboratory of the Anatomy Department, Universiti Kebangsaan Malaysia (Cheras, Malaysia), at an ambient temperature of 25 ± 3 °C and 12-h light/dark cycle throughout the study. This study followed the guidelines of the Institute for Laboratory Animal Research, National Research Council, *Guide for the Care and Use of Laboratory Animals (1996)* [15]. The Animal Ethics Committee at Universiti Kebangsaan Malaysia reviewed and approved the study procedures (approval code: ANAT/PP/2017/FAIRUS AHMAD/27-SEPT./871-OCT.-2017-SEPT.-2018).

Study design

The rats were group equally into the normal control, negative control (on HCHF diet), and honey group (n = 8) (on HCHF diet and honey supplementation). Standard rat chow and tap water were given to the rats in the normal control group. For 16 weeks, the negative control and honey groups were given an HCHF diet with fructose-enriched drinking water (25 mg/100 mL). From Week 8 to the end of the experiment, the honey group was orally administered 1g/kg of KH daily. After 16 weeks, rats were euthanised with ketamine/xylazine/zoletil (KTX) mix and left, and right femurs were harvested and cleaned. Neutral buffered formalin was used to preserve right femurs, while the left femurs were kept at -80°C.

Micro-computed tomography

A micro-CT device was used to determine micro-architectural parameters of the bone (cortical and trabecular bone parameters) (Skyscan 1076 scanner, Scanco Medical, Switzerland). The left femur was placed in the sample holder before scanning, and the following settings were used: X-ray voltage of 70 kV, X-ray current of 100 μA, 0.5 mm aluminium filter, 0.7° rotation step, image pixel size of 9 μm, medium camera resolution, and frame averaging of 3. The integration time was set to 200 milliseconds. The software NRecon was used to reconstruct the slides (V1.6.10.4, Skyscan, Belgium). The trabecular volume of interest (VOI) began at 1.0 mm, while the cortical VOI began at 5.0 mm from the proximal growth plate and extended to the tibial distal end. CTAn software (V1.16.1.0 +, SkyScan, Belgium) was used to assess the images.

Histomorphological examination of femur

The decalcified femurs were used for the histomorphometric analysis. The right femurs were soaked in 10% neutral buffered formalin for two days before being sawed into halves and subjected to 30-day decalcification in 10% ethylenediaminetetraacetic acid (EDTA). The EDTA solution was replaced three times per week. Decalcified bone samples were placed in a plastic cassette and soaked in tap water overnight before being dehydrated for 9 hours with a graded alcohol series. The dehydrated bone samples were soaked in absolute toluene overnight after 90 minutes in an alcohol and toluene (1:1) solution.

Embedding of samples in paraffin wax at 60°C was then performed. Using a microtome (Leica RM2235, Nussloch, Germany), 5 μm-thick paraffin sections were produced, placed on glass slides, cleared with xylene and rehydrated with decreasing alcohol concentrations and finally rinsed with water. Haematoxylin and eosin (H&E) stain was used to colour the slides, which were then dehydrated with increasing alcohol concentrations, and mounted with mounting media and coverslips. The slides were examined under a light microscope (Zeiss Primo Star, Germany), and images were captured at 400 magnification using the Zen 2.6 lite software. The Weibel grid technique was used to quantify bone cellular indices.

Femur three-point bend testing and analysis biomechanical testing

The mid-point of femoral diaphysis was put through the three-point bending test to estimate the skeletal mechanical properties. The femurs were thawed at room temperature before their weight, length and width were measured. Each bone was loaded from above at the mid-point on its anterior surface between two lower supports 10 mm apart. The bones were fractured by a Shimadzu Universal Testing Machine (Autograph AGS-X 500N, Japan) that applied force at a speed of 5 mm/min onto the bone surface. Trapezium X software analysed the input from the machine and generated the biomechanical parameters.

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) version 26 (IBM, Armonk, NY, USA). The intergroup differences of bone micro-architectural and biomechanical parameters were determined using one-way analysis of variance with Tukey HSD. All data were presented in mean ± standard error. Statistical significance was set at $p < 0.05$.

RESULTS

Micro-architectural indices of rats

Total cross-sectional area (Tt.Ar) was significantly decreased in the negative control group compared to the normal control ($p < 0.05$). Tt.Ar of the honey supplemented group was not different statistically from the normal control and negative control ($p < 0.05$). The differences in other cortical bone parameters among the experimental groups were not significant statistically ($p > 0.05$). All trabecular parameters did not differ significantly among the experimental groups ($p > 0.05$). (Table 2). Figure

1 shows three-dimensional images of the microstructure of the bone. No apparent difference in the trabecular and cortical bone structure among the groups could be observed.

Biomechanical characteristics

Displacement and strain were significantly reduced in the negative control group than the normal control group ($p < 0.05$). However, the displacement and strain of the honey supplemented group were not significantly different with the normal control and positive control groups ($p > 0.05$). All study groups showed no significant differences in load, stress, stiffness and Young's modulus of elasticity ($p > 0.05$). (Table 3).

Histomorphology of the femur

Figure 2 shows H&E-stained photomicrographs. Compared to the normal control group, ES/BS was significantly increased while OS/BS and OV/BV were significantly reduced in the negative control group ($p < 0.05$).

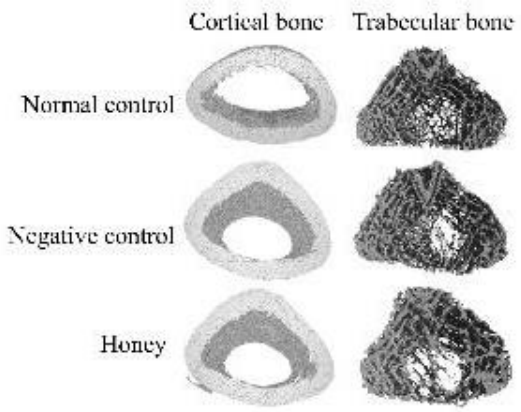

Figure 1: 3D images of trabecular and cortical bone obtained from micro-CT scanning in the normal control, negative control and Kelulut honey-treated rats at the conclusion of the experiment

Table 2: Microstructural indices of the rats subjected to different treatment

Microstructure index	Abbreviation	Normal control	Negative control	Honey
Trabecular bone				
Trabecular Thickness (mm)	Tb.Th	0.0579 ± 0.008	0.0408 ± 0.006	0.0483 ± 0.003
Trabecular Seperation (mm)	Tb.Sp	0.238 ± 0.060	0.106 ± 0.020	0.162 ± 0.041
Trabecular Number (1/mm)	Tb.N	4.04 ± 0.58	3.85 ± 0.70	4.50 ± 0.38
Bone Volume Fraction (%)	BV/TV	21.2± 1.8	17.7 ± 3.0	21.1 ± 1.1
Connectivity Density (1/mm^3)	Conn.D	2953 ± 759	3288 ± 659	3356 ± 455
Structural Model Index	SMI	1.63 ± 0.25	1.82 ± 0.27	1.62 ± 1.31
Cortical bone				
Total Cross-sectional Area (mm^2)	Tt.Ar	13.8 ± 1.1	9.18 ± 1.42*	10.4 ± 0.5
Cortical Area (mm^2)	Ct.Ar	2.97 ± 0.29	2.00 ± 0.39	2.31 ± 0.18
Cortical Area Fraction (%)	Ct.Ar/Tt.Ar	21.6 ± 2.0	18.7 ± 3.2	22.1 ± 1.1
Cortical Thickness (mm)	Cr.Th	0.219 ± 0.077	0.0618 ± 0.009	0.0634 ± 0.005

*The mean ± standard error of the mean (n = 8 rats in each group) is used to express the results. One-way analysis of variance with Tukey HSD pairwise comparison was used to determine statistical significance. *P < 0.05 vs. the control group*

Table 3: Femoral biomechanical strength of the rats subjected to different treatment

Parameter	Normal control	Negative control	Honey
Femur length (mm)	39.1 ± 0.7	32.2 ± 4.6	36.2 ± 0.6
Femur width (mm)	4.63 ± 0.11	3.82 ± 0.55	4.43 ± 0.12
Femur weight (g)	0.863 ± 0.078	0.538 ± 0.129	0.825 ± 0.073
Load (N)	116 ± 12.1	81.0 ± 16.1	93.5 ± 8.0
Displacement (mm)	5.20 ± 0.23	3.15 ± 0.63**	4.36 ± 0.25
Stiffness (N/mm)	22.5 ± 2.4	23.7 ± 4.8	21.4 ± 1.2
Stress (Nmm-2)	631 ± 66	465 ± 93	524 ± 36
Strain (%)	5.27 ± 0.23	3.23 ± 0.67**	4.27 ± 0.22
Young's Modulus of elasticity	119 ± 13	134 ± 27	123 ± 7

*The mean± standard error of the mean (n = 8 rats in each group) is used to express the results. One-way analysis of variance (ANOVA) with Tukey HSD pairwise comparison was used to determine statistical significance; **p < 0.01 vs normal control group*

Table 4: Static bone histomorphometric parameters

Bone static index	Abbreviation	Normal control	Negative control	Honey
Osteoblast surface/bone surface (%)	Ob.S/BS	0.119 ± 0.042	0.026 ± 0.013	0.039 ± 0.021
Osteoclast surface / bone surface (%)	Oc.S/BS	0.110 ± 0.028	0.076 ± 0.022	0.012 ± 0.012**
Eroded surface/bone surface (%)	ES/BS	0.206 ± 0.047	0.404 ± 0.041*	0.331 ± 0.057
Osteoid surface/bone surface (%)	OS/BS	0.498 ± 0.059	0.078 ± 0.022***	0.272 ± 0.077*
Osteoid volume/bone volume (%)	OV/BV	0.192 ± 0.035	0.050 ± 0.026*	0.112 ± 0.034

*The mean ± standard error of the mean is used to express the data (n=8/group). One-way analysis of variance with Tukey HSD pairwise comparison was used to determine statistical significance. *p < 0.05, **p < 0.01, ****p < 0.001 vs. control group*

Oc.S/BS and OS/BS were significantly reduced in the honey group ($p < 0.05$) than the normal control group. Across all treatment groups, the Ob.S/BS was similar ($p > 0.05$). (Table 4).

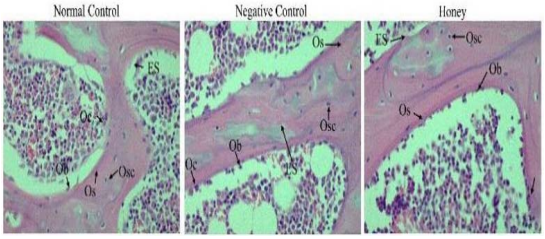

Figure 2: Representative micrographs of decalcified trabecular bone stained with haematoxylin and eosin from the normal control, negative control, and honey-treated groups (X400 magnification). Abbreviations: ES, eroded surface; Ob, osteoblast; Oc, osteoclast; Os, osteoid; Osc, osteocyte

DISCUSSION

This study showed marginal deterioration of bone health induced by MetS, as evidenced by significant reduction in Tt.Ar, OS/BS, OV/BV, displacement and strain in the negative control group compared to the normal control group. The ES/BS also increased in the negative control group in comparison to normal control group. Other microstructural and biomechanical parameters were comparable in rats receiving standard diet and HCHF diet. KH supplementation increased OS/BS and reduced Oc.S/BS in rats fed with HCHF. However, it did not reverse other bone-damaging impacts of HCHF diet.

Micro-computed tomography provides a high-resolution, three-dimensional view of trabecular and cortical bone structure. This technique serves as a benchmark for skeletal geometry evaluation [16]. The minimum parameters required to describe trabecular bone micro-architecture are BV/TV, Tb.N, Tb.Th, and Tb.Sp, whereas the minimum parameters to describe cortical bone micro-architecture are Tt.Ar, Ct.Ar, Ct.Ar/Tt.Ar, and Ct.Th [17]. The micro-architecture of trabecular and cortical bone is critical in determining bone quality and strength [18].

Only Tt.Ar deteriorated in rats fed an HCHF diet in this study, while the other micro-architecture parameters were unaffected. This finding contradicts that of a previous study. Wong *et al* [4] reported a significant deterioration of trabecular microstructure, Ct.Ar and Tt.Ar, in rats receiving HCHF diet of similar composition in our study after 20 weeks. Since the rats in the present study were given HCHF diet for 16 weeks only, the negative effects might be attenuated. In another study, male Sprague Dawley rats fed with a high-fat diet (HFD) for 22 weeks showed significant deterioration of trabecular bone. Expression of osteoblast markers such as alkaline phosphatase and total procollagen type 1 N-terminal propeptide was reduced, but osteoclast bone resorption marker, i.e. C-terminal telopeptide, decreased in HDF-fed rats [19]. In the study by Lac *et al* [20], male Wistar rats fed with HFD for 10 weeks showed a significant reduction in bone mineral density, bone mineral content and skeleton. In contrast, Yanagihara *et al* [21] reported increased bone mineral density in female Wistar rats fed with HFD for 5 weeks. However, there were no corresponding changes in microstructural and mechanical bone properties in these rats.

In this study, KH did not reverse the deterioration of Tt.Ar caused by HCHF diet. It also did not alter other trabecular and cortical microstructural indices in the rats. This observation contradicted previous studies. In a study by Zaid *et al* [9], ovariectomised (OVX) female rats treated with 200 mg/kg of Tualang honey for 6 weeks showed a significant increase in trabecular microstructure than the OVX control group. Kamaruzzaman *et al* [22] demonstrated that male rats with bone loss induced by glucocorticoids treated with 0.2 g/kg of KH for eight weeks exhibited a substantial increase in trabecular bone microstructure than the negative control group. Since the rats in this

study did not suffer substantial bone microstructure loss due to HCHF diet, we conclude that KH did not improve skeletal microstructure in rats without prior bone damage.

Biomechanical properties of bone can be classified into extrinsic properties (load, stiffness and displacement) and intrinsic properties (stress, strain and Young's modulus of elasticity). The extrinsic properties are influenced by the density, architecture and tissue properties of the bone, while the intrinsic parameters are independent of the amount of bone in the tissue [23]. Load and stress measure the maximum force needed to create bone fracture (bone strength). Stiffness and Young's modulus of elasticity show the resistance of the bone against elastic deformation. Displacement and strain measure the ability of the bone to undergo significant deformation before fracture (ductility) [23]. In this study, rats receiving HCHF showed reduced ductility indicated by reduced displacement and strain than the normal control group. Wong *et al* [5] reported that male rats given HCHF diet for 20 weeks showed decreased load but increased displacement and strain. Therefore, prolonged consumption of HCHF diet may reduce bone strength.

In this study, KH supplementation did not enhance bone strength in rats fed with HCHF diet. This result contradicted previous studies, which found that honey supplementation increased bone strength. According to Yudaniayanti *et al* [24], 3-month-old OVX female rats supplemented with honey (100, 200 and 400 mg/kg) for 12 weeks showed increased bone strength. Several studies have also reported that honey supplementation (100 mg/kg) in female Sprague Dawley rats (8 and 16 weeks) increased trabecular bone micro-architecture and biomechanical properties [25–27].

Static bone histomorphometric parameters provide a snapshot of bone remodelling activity by clarifying the quantity of unmineralised bone (osteoid) and degree of resorption cavities in the trabecular bone [28]. In this study, rats with MetS showed a significant decrease in OV/BV and OS/BS, and a significant increase in ES/BS. These observations were comparable to a previous study by Wong *et al* [4]. The significant reduction in OS/BS and OV/BV suggests that HCHF reduced bone formation. The increase in ES/BS signifies that the HCHF diet increased bone resorption. This study suggests that 16 weeks of HCHF diet is enough to impair bone formation. Wang *et al* [29] showed decreased osteoblast differentiation in mesenchymal stem cells exposed to high glucose level.

In this study, KH group showed decreased OS/BS and Oc.S/BS than the normal control group. In contrast, Kamaruzzaman *et al* [22] showed that male rats with glucocorticoid-induced bone loss and treated with 0.2 g/kg of KH for eight weeks experienced an increase in Ob.S/BS and a reduction in Oc.S/BS than the negative control group. These findings suggest that while KH did not improve bone formation, it did reduce the number of osteoclasts. However, it will take more time for these cellular changes to affect the bone structure.

Several studies have explored the impact of MetS on skeletal health. Obesity protects the skeleton via increased mechanical loading on the bone. Adipose tissue also secretes hormones that stimulate bone formation, such as oestradiol, insulin, amylin and preptin. On the other hand, increased adipocyte differentiation hinders osteoblast differentiation in the bone marrow because they originate from the same progenitor (mesenchymal stem cell) [30]. Pro-inflammatory cytokines also originate from adipose tissue. They could upregulate receptor activator of NF-κB (RANK)/RANK ligand pathway, leading to osteoclast differentiation [31]. Excess urinary calcium secretion leads to secondary parathyroidism in hypertensive conditions, which can accelerate osteoporosis by increasing calcium mobilisation from the bone [32]. Hyperglycemia promotes the production of reactive oxygen species, which enhances osteoclastogenesis and bone resorption activity, and subsequently, bone loss [33]. All these mechanisms could have contributed to the deterioration of bone health of rats receiving the HCHF diet, but KH did not reverse the skeletal adverse effects.

Several limitations of this study need to be addressed. The primary aim of this research was to determine the effects of KH in reversing MetS and these findings have already been published [7]. The dose and treatment period might not be optimal to produce significant skeletal effects, considering that it takes more time for cellular changes to be reflected on bone structure. In future studies, the dose and duration of KH treatment could be increased and longitudinal follow-up of the skeletal parameters could be performed to address this limitation. The molecular response of the bone towards KH supplementation was also not examined in this study. HCHF diet is known to induce inflammation in rats, which could potentially exert negative effects on the skeletal system [4]. As a result, future research should include relevant markers to study the influence of MetS inflammation on skeletal health.

CONCLUSION

MetS reduces bone size and compromises bone strength. Kelulut honey did not reverse HCHF diet-induced bone loss. More comprehensive studies on bone cellular and homoeostasis markers should be performed to validate these findings.

DECLARATIONS

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Conflict of interest

This work has no potential conflicts of interest.

Contribution of authors

We declare that this work was completed by the authors named in this article, and that the authors will be held liable for any claims relating to the content of this article. The manuscript was read and approved for publication by all authors. The study was conceived and designed by Fairus Ahmad and Kok-Yong Chin, with data collected and analysed by Nur Zuliani Ramli, Khairul Anwar Zarkasi, and Sophia Ogechi Ekeuku, and the manuscript written by Sophia Ogechi Ekeuku. Kok-Yong Chin and Fairus Ahmad secured funding of the study and provided critical review to the manuscript.

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