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Potential benefical effects of stingless bee honey (kelulut honey) on bones exposed to long-term dexamethasone

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ABSTRACT

Osteoporosis due to chronic exposure to glucocorticoids (GCs) is related to oxidative stress and is the leading cause of secondary osteoporosis. Stingless bee honey or 'kelulut honey' (KH) has been proven to have antioxidant properties. The aim of this study was to determine the potential benefit of KH in protecting the bone against chronic glucocorticoid therapy. Fourty eight adult male Sprague-Dawley rats, aged 3 months weighing 280-300 g were used in this study. Thirty two rats underwent adrenalectomy and were divided into four groups of eight. They were administered with 120 µg/kg/day of dexamethasone intramuscularly (AC) and supplemented with oral KH 200 mg/kg/day (KH200), 400 mg/kg/day (KH400) and 2% calcium in drinking water (PC). The AC group was given oral normal saline 0.1 ml/kg/day. Eight rats underwent sham procedure and were given vehicle palm olein 0.05 ml/kg/day intramuscularly and oral normal saline 0.1 ml/kg/day (Sham). The baseline control rats (BL) were euthanized without receiving any treatment. The rats were euthanized after two months of treatments. The results showed that AC had significantly lower osteocalcin and Cross Linked C-Telopeptide of Type 1 Collagen (CTX) with reduction in bone biomechanical strength and bone mineral density (BMD). KH200 and PC groups had significantly higher osteocalcin level and KH400 had lower CTX level compared to AC. KH200 and PC groups had higher biomechanical strength parameters (Modulus and stress) and BMD compared to the AC group. However, KH supplementation at both doses did not prevent changes to the BMD. The findings suggested that KH has potential benefits in protecting the bone against glucocorticoid-induced osteoporosis.

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INTRODUCTION

Osteoporosis is characterized by microarchitectural deterioration of bone tissue and decreased bone mass, leading to bone fragility and increased risk of fracture (NIH, 2001). Approximately 200 millions of global population suffered from osteoporotic fracture with expending prevalence (Li et al., 2012). Glucocorticoids (GCs) are frequently used for the treatment of inflammatory and chronic diseases. However, clinical studies have shown that, administration of GCs for more than six months is associated with osteoporosis in 50% of

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users (Winblad et al., 2017; Adinoff and Hollister, 1983). However the risk of glucocorticoid-induced osteoporosis (GIO) depends on the cumulative dosage in which greater than or equal to 1000 mg is more strongly associated with fractures compared to smaller cumulative dose (<1000 mg) (Adami and Saag, 2019). GIO is the most common cause of secondary osteoporosis particularly affecting young individuals (Langdahl and Ralston, 2017; Buckley et al., 2017). The incidence of fracture within the young GCs users is approximately 30% after 5 years of treatment which mainly involves the vertebrae, with increasing incidence of non-vertebral fracture such as the neck of the femur (Skoner, 2016). Osteoporosis is a major limiting factor in the long term GCs treatments which is not overcome by the co-prescription of bisphosphonates (Weinstein et al., 2002). A study also found that GCs caused impairment of skeletal metabolism and aggravated the osteoporosis due to aging (Rinne et al., 2017).

GCs treatment causes a decrease in bone formation (Kang et al., 2016; Frenkel et al., 2015; Geurtzen et al., 2017). GCs upregulates the peroxisome proliferator-activated receptor gamma receptor 2 (PPARγ2) (Ito et al., 2007) and affects the Wnt/β-catenin signaling pathway (Ohnaka et al., 2005) that lead to reduction in the osteoblast number. GCs increase the expression of sclerostin, which inhibits Wnt signaling, reducing the differentiation of osteoblast precursors to mature osteoblasts (Sato et al., 2016). It was shown in animal models that GCs also affect osteocyte morphology and mineralization (Swanson et al., 2006).

The direct effects of GCs on bone resorption is mediated by the increase in production of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) and decrease production of osteoprotegerin (OPG) which increase both the osteoclast number and activities (Lane et al., 2006; Hofbauer et al., 1999). Studies have proven that fracture occurs at a higher BMD in individuals receiving GCs compared to non-glucocorticoid treated people (Luengo et al., 1991; Van Staa et al., 2004; Kanis et al., 2004). GCs also cause disruption to the tracecular bone structure (Sutter et al., 2014).

GIO is under recognized and treated based on the studies done in the United States (Curtis et al., 2005; Feldstein et al., 2005). Currently, oral bisphosphonates are widely used as the first line bone protective therapy. However, teriparatide therapy showed significant increase in spine and hip BMD and reduced vertebral fracture risk in osteoporosis induced by GCs (Langdahl et al., 2009).

Honey is documented as one of the most ancient traditional remedies in history (Molan, 2006; Simon et al., 2009). Honey contains mainly carbohydrate with fructose being the highest component which account for about 32–38% followed by glucose and other disaccharides and oligosaccharides (Rao et al., 2016; Bogdanov et al., 2008). The content of fructose in honey however has greater long term benefits for improving glycemic control (Cozma et al., 2012). Other components of honey include organic acids, minerals, trace elements, numerous vitamins, enzymes and proteins, flavanoids and phenolic acids (Rao et al., 2016; Bogdanov et al., 2008; Solayman et al., 2016; Saba et al., 2013). These chemical constituents of honey makes it beneficial in human health.

The phenolic acids and flavonoids are responsible for the well-established antioxidant activity of honey. Researchers also showed that honey (1.2 g/kg) elevated the amount and activity of antioxidant agents (Mohd Norowi et al., 2008; Kishore et al., 2011). Apart from these, antioxidant effects of honey are also contributed by the sugars, proteins, amino acids, carotenes, organic acids, Maillard reaction products, production of reactive oxygen species (ROS), and other minor components (Gheldof and Engeseth, 2002; Aljadi and Kamaruddin, 2004). The exact antioxidant mechanism is unknown, but the proposed mechanisms include free radical sequestration, hydrogen donation, metallic ion chelation, flavonoids substrate action for hydroxyl, and superoxide radical actions (Al-Mamary et al., 2002; Van Acker et al., 1996).

Stingless bee is a natural type of bee that exists in almost every continent. They are easier to handle, compared to other honey bees which contributes to easy availability of the honey (Khairunnisa, 2011). Likewise, honey produced by stingless bee is unique as it originates from the rich vegetation in native environments. It has a distinctive sweetness mixed with a sour and acidic taste. Stingless bee honey or also known as ‘kelulut honey’ (KH) has been proven to accelerate wound healing through its antioxidant properties. A study had found that KH has higher flavonoids content compared to other honeys and its content of polyphenols is the highest compared to other South American honey. Apart from its antioxidant effect, KH also exhibit anti-inflammatory and antimicrobial activities. There is still limited knowledge about this honey, which makes it less popular compared to other honey (Guerrini et al., 2009). Therefore, KH should be further explored due to its mass production and convenience of management. To date, there is paucity of literature on the effects of honey or KH on osteoporosis. Recently, Tualang honey has been studied for its effect on bone density and a positive result was exhibited (Zaid et al., 2012). This could make an opening view for the potential benefits of honey as an alternative treatment for osteoporosis, in conjunction with the conventional treatments. This study explored the effects of KH on bone density and its anti-osteoporotic properties on osteoporosis induced by prolonged glucocorticoid administration, considering oxidative stress involvement in its pathophysiology.

MATERIALS AND METHODS

The protocol of animal experimentation and treatment were reviewed and approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the number ANAT/PP/PP/2016/ELVY/28-SEPT/789-OCT.-2016-SEPT-2018.

Animal treatments

Forty eight male Sprague-Dawley rats aged 12 weeks old weighing between 280 and 300 g were obtained from the Laboratory Animal Resource Unit of Universiti Kebangsaan Malaysia. The rats were kept in plastic cages in the animal house of Department of Anatomy, Universiti Kebangsaan Malaysia (Cheras, Malaysia) with a constant ambient temperature of 25 ± 2°C and an alternated 12 h light/dark cycle. Upon acclimatization for one week, the rats were divided into six groups (n=8 per group): four controls and two test groups. The four control groups were i) baseline control (BL), ii) sham-operated (Sham), given normal saline 0.1 ml/100g/BW via oral gavage, iii) dexamethasone treated adrenalectomized-control (AC), given normal saline at 0.1 ml/100g/BW via oral gavage, and dexamethasone intramuscularly at the dose of 120 µg/kg/day iv) dexamethasone treated adrenalectomized rats supplemented with 2% calcium as *ad libitum* and dexamethasone intramuscularly at the dose of 120 µg/kg/day (PC). This study used two test arm groups to test two different doses of KH; i) dexamethasone treated adrenalectomized rats supplemented with KH 0.2 mg/kg/BW via oral gavage (KH200) and ii) dexamethasone treated adrenalectomized rats supplemented with KH 0.4 mg/kg/BW via oral gavage (KH400). Both groups received dexamethasone intramuscularly at the dose of 120 µg/kg/day. The adrenalectomy procedure was done under general anesthesia. The Sham rats underwent similar procedure, except that the adrenal glands were not excised and left *in-situ*. All adrenalectomized groups were given dexamethasone intramuscularly at the dose of 120 µg/kg/day. The dose and duration of dexamethasone treatment used was based on a previous study (Elvy Suhana et al., 2011). Instead of dexamethasone, the Sham rats were given vehicle normal saline 0.05 ml/kg/day intramuscularly. KH was obtained from Kelulut Honey Farm (Sg Pusu Gombak) Malaysia.

Prior to adrenalectomy, the rats were anesthetized using 90 mg/kg ketamine (Troy Laboratories, Pty Ltd, Australia) and 10 mg/kg xylazil (Troy Laboratories, Pty Ltd, Australia). The surgical wounds were cleaned and poviderm cream was applied daily until completely healed. The BL rats were sacrificed at the commencement of the experiment to obtain the baseline bone mechanical strength parameters and the pre-treatment level of bone biomarkers. The adrenalectomized rats were given normal saline *ad libitum* to maintain the sodium homeostasis after removal of the adrenal glands. The administration of test and control materials was started at day 14 post-surgery by oral gavage (9:00-10:00 am daily for six weeks). The animal grouping and treatments are summarized in a flowchart in Figure 1.

Several criteria were considered prior to euthanized, such as animal current quality of life (pain, distress, diseased etc), appropriate euthanasia method identified and any altered behaviour that could intefere with research findings. The death of the rats were verified by examining the animal for cessation of vital signs (respiratory rate, heart rate, peripheral perfusion). The rats were sacrificed 24 h after the last dose of treatment under excess of ketamine/xylazil anesthesia (300 mg/kg) at the ratio of ketamine:xalazil 90:10 after completing two months' treatment. The right femur was collected, wrapped in gauze soaked with phosphate-buffered saline (PBS) and kept in -80°C until analyzed.

Along the study, the rats were monitored daily as routine weight measurement was taken. Furthermore the general activity and behaviour of rats were also observed in case of changes due to corticosteroid influence. If these change occured, the rats were excluded since it could alter the research findings. All animal welfare considerations were taken by doing the appropriate observations to the animals by evaluating five aspects of an animal's condition (Canadian Council of Animal Care, 1998): i) behavioral responses to external stimuli, ii) changes in unprovoked behavior iii) measurable clinical signs (respiratory rate) and iv) external physical appearance. Total of 8 animals died during the course of treatments in this study: 2 of them due to aspiraton, systemic effect of corticosteroid – 3, post surgical complication – 3.

Measurements of body composition and bone densitometry

The rats were anaesthetized under general anesthesia using ketamine/xylazil while conducting bone densitometry procedure. The anaesthetized rat was placed in ventral recumbence position on the scan table. The scanning procedure was performed using dual-energy X-ray absorptiometry (DXA) (Discovery DXA System, Hologic Canada) to evaluate body composition (percentage of fat and BMD) before the rats were euthanized. All the parameters were analyzed using Small Animal Analysis Software, Hologic QDR-1000 System software.

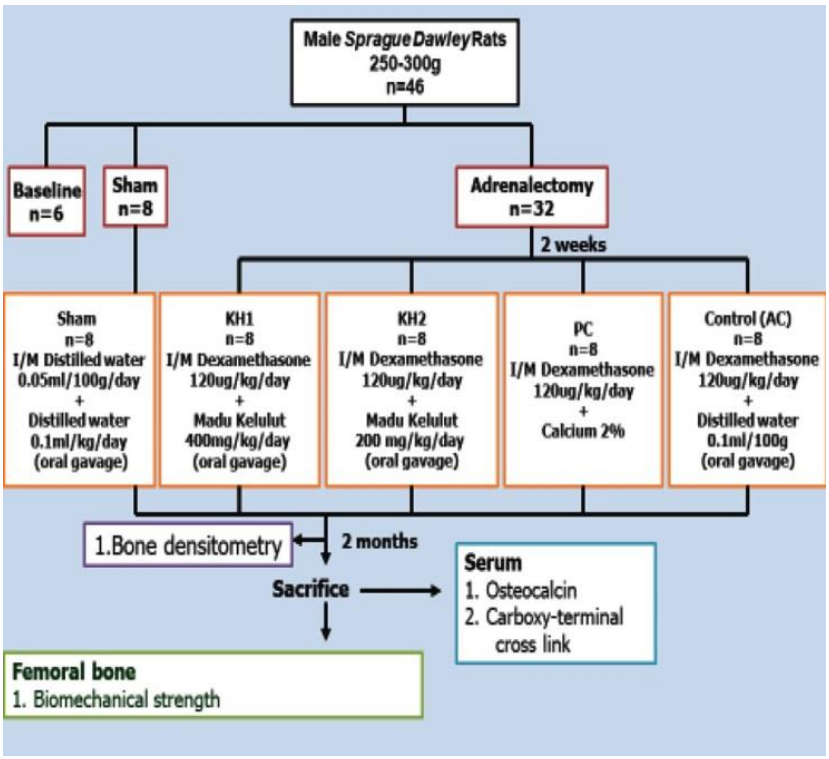

Figure 1. Schematic diagram of the division and treatment of rats from each experimental group.

Biochemical assay for bone markers

Osteocalcin (bone formation marker) and CTX-1 (bone resorption marker) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kit as per manufacturer's instructions (Immunodiagnostic Systems, Tyne and Wear, UK).

Biomechanical strength

A three-point bending test was conducted to evaluate the biomechanical strength of the femur. A precision universal tester (Autograph AG-10kNG; Shimadzu) with Trapezium X materials testing operation software was used for this procedure. The femur cleaned of soft tissues was placed on an aluminum base; with the dorsal proximal femur placed on the one-rounded edge-free notch. The distal diaphysis at the synostosis was position on the opposite side. The femur was moistened with PBS throughout the test. The roller stamp with the tip consisting of axle-led aluminum was driven down at the central femoral diaphysis (speed 5 mm/min; span length 10 mm) until the primary strength of 1 N was achieved. The test ended automatically once a loss of force >20 N or a linear change of 2 mm was detected upon fracturing of the femur at the midshaft to avoid bursting of the specimens. The analysis of load (N), displacement (mm), stress (N/mm^2), strain (%), and elasticity (N/mm^2) was performed using the Trapezium X software.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) version 22 software. Data were tested for normality using the Kolmogrov-Smirnov test and all data were normally distributed. The data were analysed using parametric statistics, analysis of variance (ANOVA) test followed by the post-hoc Tukey test for multiple comparisons between the treatment groups. The p values <0.05 was taken as significant. Data was presented as mean ± standard error of the mean (SEM).

RESULTS

Bone biochemical markers

The circulating osteocalcin level showed a significant decrease after two months compared to the pre-treatment level in the Sham and the AC groups ($p<0.05$).

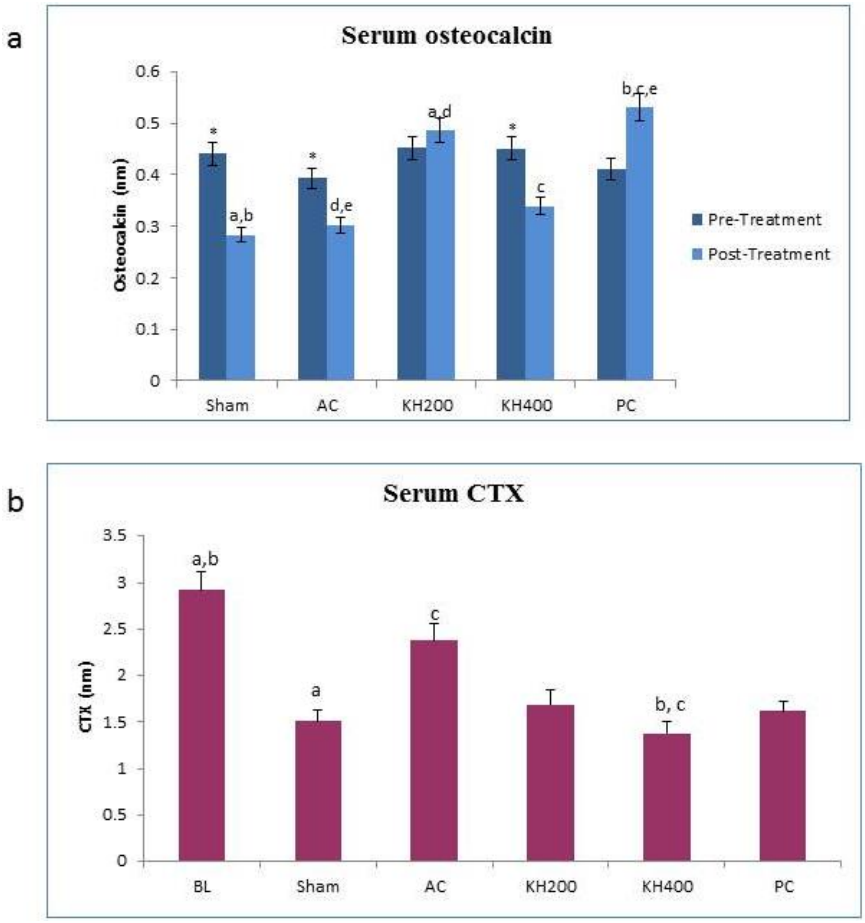

Figure 2. Serum osteocalcin (a) and CTX (b). Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p< 0.05. **BL,** Baseline control; **Sham,** sham operated control; **AC,** adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); **KH200,** adrx and given intramuscular dexamethasone 120 µg/kg/day and kelulut honey 200 mg/kg/day; **KH400,** adrx and given intramuscular DEX 120 µg/kg/day and oral kelulut honey 400 mg/kg/day; **PC,** adrx and given intramuscular dexamethasone 120 µg/kg/day and calcium 2%.

Decrease in the post-treatment osteocalcin level was also seen in the KH400 group while the KH200 and PC groups showed a significant increase to the osteocalcin levels after two months compared to the pre-treatment level. The post treatment osteocalcin levels in the KH200 and PC group were also found to be significantly higher compared to the post-treatment level of the Sham and the AC group. Post-treatment osteocalcin levels of the Sham, AC and the KH400 groups were not significantly different (Figure 2a).

The circulating CTX-1 level was significantly increased after two months in the AC rats compared to the Sham and the KH200, KH400 and PC rats showed significant lower CTX levels compared to the AC group at the end of two months ($p<0.05$). KH200 and PC group also showed a decrease in the CTX level compared to AC but did not achieve significant values (Figure 2b).

Body composition and bone densitometry

Results of BMD obtained from DXA scan revealed that rats treated with dexamethasone (AC) had significantly lower BMD in the left femur compared to the Sham rats. However, BMD of the entire body were not significantly different amongst the groups. Supplementation of honey in both higher (KH400) and lower dose (KH200) did not result in any significant changes in the BMD. However, the calcium supplemented rats (PC) showed a significant increase in the BMD (Figures 3a and b).

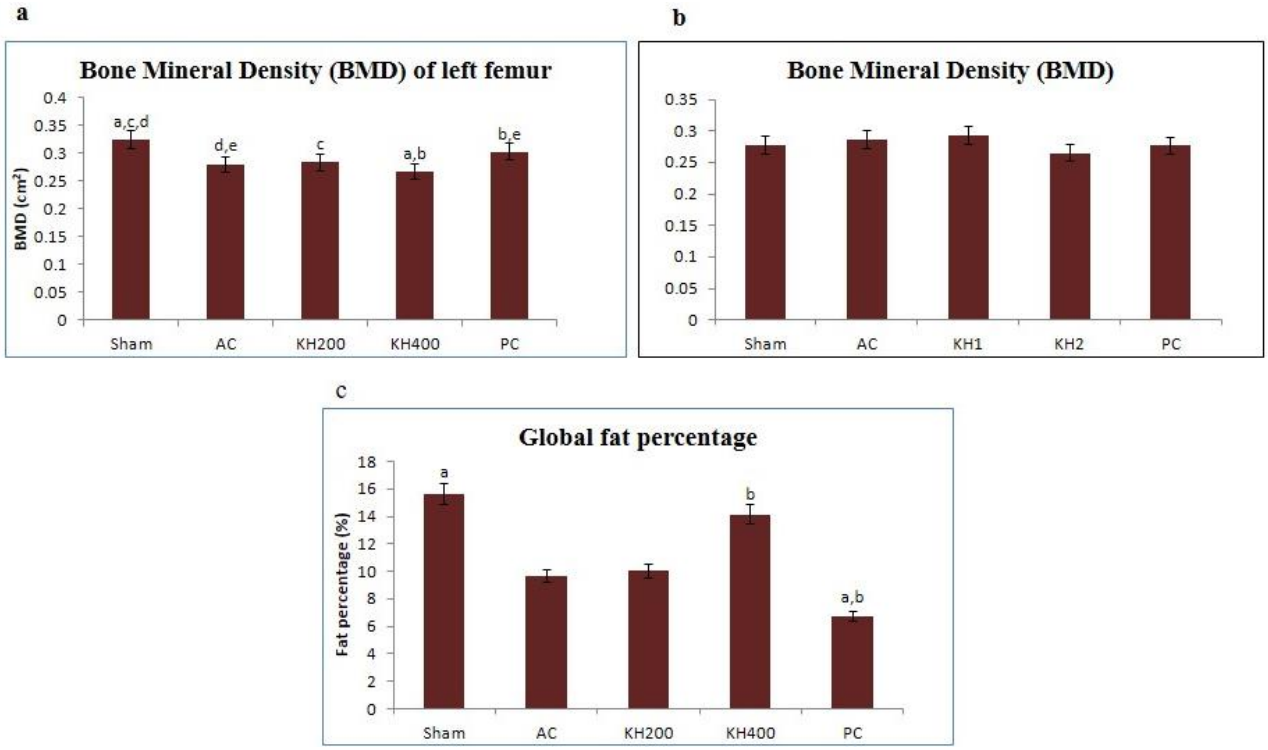

Figure 3. Bone mineral density (a), Bone mineral density of the left femur (b) and Global fat percentage (c). Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p< 0.05. **Sham,** Sham operated control; **AC,** adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day (Dex); **KH200,** adrx and given intramuscular dexamethasone 120 µg/kg/day and kelulut honey 200 mg/kg/day; **KH400,** adrx and given intramuscular dexamethasone120 µg/kg/day and oral kelulut honey 400 mg/kg/day; **PC,** adrx and given intramuscular dexamethasone 120 µg/kg/day and calcium 2%.

The global fat percentages were comparatively lower in the AC rats compared to the Sham rats but it was not statistically significant. Global fat percentage of the KH200 rats was not significantly different from the AC group. However, supplementation of KH at higher dose (KH400) had significantly increased the fat percentage compared to AC group and the rats that received 2% calcium supplementation (PC) had significantly lower global fat percentage compared to the Sham and KH400 groups (Figure 3c).

Bone biomechanical strength

Dexamethasone had decreased the biomechanical strength of the bone, indicated by reduction in the strain at the maximum flexure load (Figure 4b) and energy at the maximum flexure load (Figure 4d) in the AC group compared to the Sham although they did not reach significant values. PC and KH200 had significantly higher stress and modulus (Figure 4a) but other parameters did not show significant difference compared to the AC group. Supplementation of calcium was able to maintain the energy at the maximum flexure load (Figure 4d) of the bones. Supplementation of KH at 200 mg/kg/day showed better protective effects to the biomechanical strength of the bone compared to 400 mg/kg/day (Figure 4).

DISCUSSION

Long term use of steroids such as GCs is associated with a higher degree of bone loss due to increasein absorption and decrease in formation. Three indicators of bone health, that is, bone biochemical markers, BMD, and biomechanical strength were evaluated in this study. Honey supplementation had shown some improvements to the bone formation and resorption markers and the biomechanical strength of the bone. This showed an indication that KH has potential protective effects against osteoporosis induced long term glucocorticoid administration. This study used rats which were three months old as rats are considered adult when they are between 8 weeks to six months old (Sengupta, 2013). Two doses of KH were tested in this study, that is, 0.4 g and 0.2 g per kg body weight. However, the lower dose of

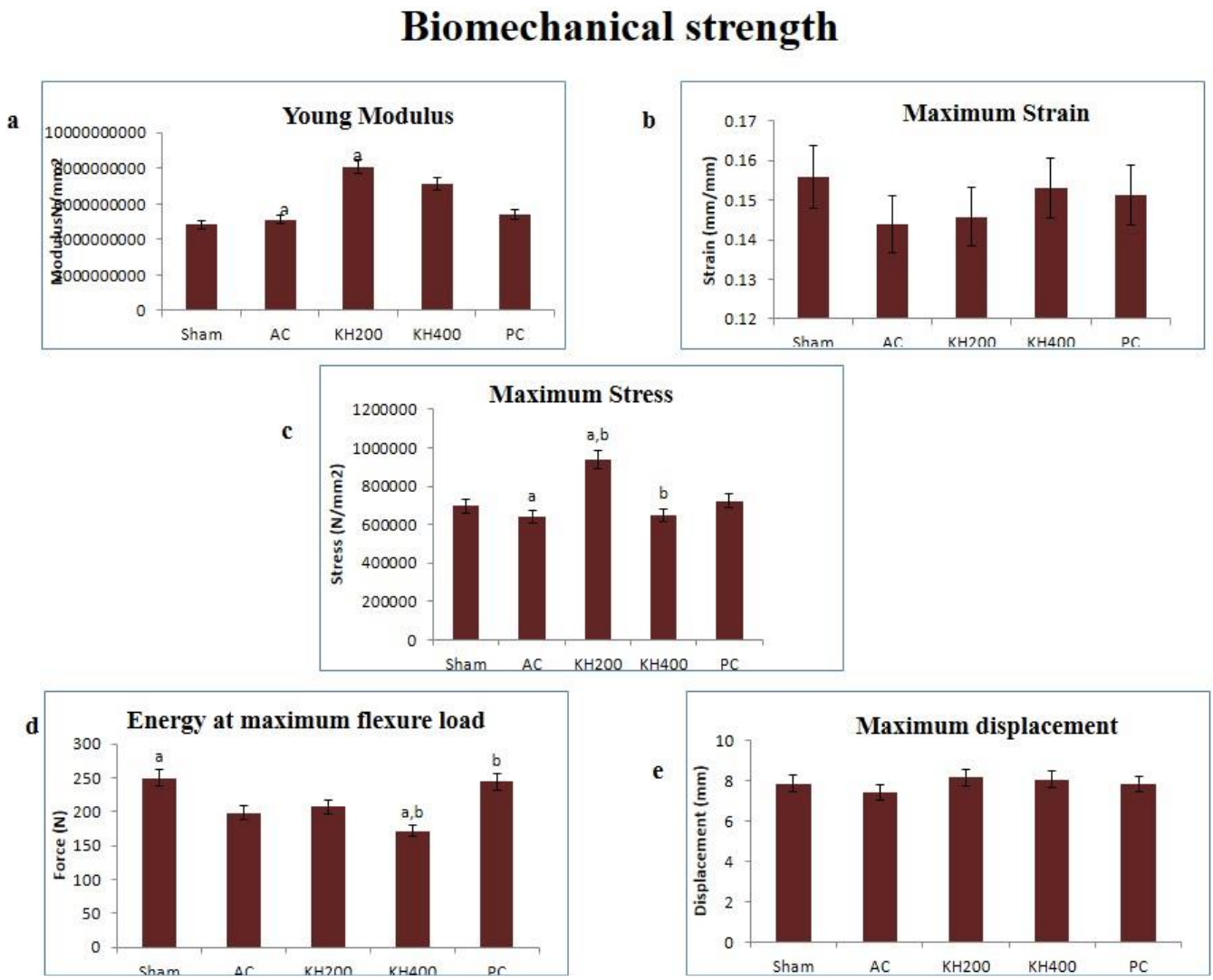

Figure 4. Intrinsic [modulus (a), maximum strain (b), maximum stress (4c)], extrinsic parameters [energy at maximum flexure load (d) and maximum displacement (4e)] of bone biomechanical strength. Data presented as mean ± SEM. Same alphabets indicate significant difference between treatment groups at p< 0.05. **Sham,** Sham operated control; **AC,** adrenalectomized (ADRX) and given intramuscular dexamethasone 120 µg/kg/day; **KH200,** adrx and given intramuscular dexamethasone 120 µg/kg/day and kelulut honey 200 mg/kg/day; **KH400,** adrx and given intramuscular dexamethasone 120 µg/kg/day and oral kelulut honey 400 mg/kg/day; **PC,** adrx and given intramuscular dexamethasone 120 µg/kg/day and calcium 2%.

honey showed better protective effects in maintaining the bone from the deleterious effects of dexamethasone. In previous study, osteoporosis induced by similar dose and duration of dexamethasone was confirmed by the changes in the structural histomorphometry parameter analysis (Elvy Suhana et al., 2011).

Two months dexamethasone treatment caused an increase to the CTX level although the level did not reach significant values but the osteocalcin level did not change after two months compared to the Sham group. The osteocalcin level were significantly reduced after two months in the Sham KH400 and AC groups and this did not happen to the KH200 and PC groups. Dexamethasone also caused a significant decrease to the BMD of the left femur but not to the global percentage of body fat. This was in agreement with previous studies where dexamethasone treatment reduced the BMD and bone calcium content of adrenalectomized rats (Ima Nirwana and Suhaniza, 2004). No significant change was seen in all bone biomechanical parameters after two months of dexamethasone treatment although previous studies showed that two months dexamethasone treatment led to reduction in both intrinsic and extrinsic properties (Elvy Suhana et al., 2018). Dexamethasone at

pharmacological concentration causes rapid stimulation of bone resorption, followed by a sustained and profound suppression of bone formation (Canalis et al., 2007; Hofbauer and Rauner, 2009; Hartmann et al., 2016). Glucocorticoids at pharmacological doses inhibit osteoblastogenesis through suppression of pro-osteoblastic genes although at physiological concentration, stimulates osteoblast differentiation (Rauch et al., 2010; Ito et al., 2007). The decrease in osteocalcin level in this study indicated reduction in bone formation. A study done on the patients with congenital adrenal hyperplasia with 21- hydrolase deficiency, where these patients were in a condition with lifelong glucocorticoid excess showed a significant decrease in BMD values (Ceccato et al., 2016). GCs could have enhanced RANKL expression which stimulates formation of resorption pits and release of CTX. Increase in formation of resorption pits might occur without affecting the number or sizes of osteoclasts formed and are not associated with increased life span of osteoclasts. GCs does not cause osteoclast apoptosis or affect mRNA expression of several osteoclastic or osteoclastogenic genes. In this study, the CTX of the dexamethasone treated rats was increased that suggested an acceleration in resorption activities. The increase in the resorption which was coupled with decrease information could have caused structural changes to the bone that led to the decrease in BMD and also compromised bone properties.

The results of this study showed that supplementation of KH at 200 mg/kg had increased the bone formation marker, osteocalcin which was comparable to calcium supplementation. However, supplementation at 400 mg/kg/day of KH did not show positive effect to the osteocalcin level. KH had also showed positive effect to bone resorption marker, CTX where it was significantly reduced. However, the positive effect of KH to CTX was better seen at the dose of 400 mg/kg/day where it showed significant decrease in the bone resorption marker. The effects of KH to bone biomechanical strength parameters were not so remarkable. The stress was significantly increased with supplementation of KH at 200 mg/kg/day. However, calcium supplementation showed better value of energy and supplementation of KH at both doses did not change the energy. Supplementations of KH at both doses did not benefit other biomechanical parameters and calcium supplementation also did not show significant changes.

The BMD protective effects of KH were not obtained in this study but calcium supplementation had proven positive effects in preserving the BMD of the femur. Moreover, the percentage of body fat was also lower in the rats supplemented with calcium and KH at the dose of 200 mg/kg/day. Prolong GCs exposures are subjected to increase in visceral fat (Delivanis et al., 2018). Patients with congenital adrenal hyperplasia have lower BMD with normal body composition (Halper et al., 2018). In this study we found that two months dexamethasone treatment reduced the percentage of body fat. It could be due to administration of dexamethasone causes increase in visceral fat accumulation which leads to central obesity but it could had caused reduction in global fat percentage. Dexamethasone administration was also found to cause hyperplasia of the perirenal fat as described in previous studies (Azwan et al., 2015; Fairus et al., 2013). This has to be confirm by measurement of the abdominal circumference which was not done in this study.

Honey is rich in antioxidants, such as flavonoids and phenolic acids (Aljadi and Kamaruddin, 2004;Al-Mamary et al., 2002; Phrzynska and Biesaga, 2008; Gheldof et al., 2007). Flavonoids, such as quercetin and kaempferol, phenolic acids and antioxidant enzymes such as glucose oxidase and catalase that are found in honey have been shown to directly induce apoptosis of mature osteoclasts, thus inhibiting bone resorption. This effect is produced due to the decrease in intracellular reactive oxygen species (ROS) in osteoclasts. Apoptosis of osteoblasts induced by GC has been identified as the main cause of osteoporosis, bone loss and fractures, and the oxidative stress was found as an important contributor. Previous studies showed that dexamethasone induces excessive production of ROS, and creates an oxidative stress environment in rat hippocampal slice cultures. Oxidative stress also induces the association of FoxOs with β-catenin (Almeida et al., 2007; Essers et al., 2005), which is a critical component of the Wnt signaling pathway and indispensable for osteoblastogenesis (Rodda and McMahon, 2006). Therefore, natural or synthetic agents with antioxidant activities can antagonize GCs-induced apoptosis in osteoblasts, thus demonstrating the potential application to reverse osteoporosis. In this study, we showed that, KH might have prevented the osteoporotic effects of dexamethasone by increasing bone formation and decreasing bone resorption based on the biochemical markers result. There is possibility that these effects were achieved through its antioxidant properties by blocking ROS overproduction which is toxic to osteoblast and has protective effects to osteoclast.

Results of this study revealed that KH supplementation at lower dose showed better protective effects against GIO compared to the higher dose. This results indicated that honey has the potential to be consumed as a bone protective agent against osteoporosis in patients need to be on long term GCs therapy. This could be due to higher intake of honey that could haved induced hyperglycaemic state to the rats. However, this yet to be confirmed by serum glucose level which was not taken in this study. Diabetes itself is associated with increased risk of fracture, although T2DM is often characterized by normal or high bone mineral density (BMD). Thus, diabetes may be associated with a reduction of bone strength, that is,

not reflected in the measurement of BMD (Swanson et al., 2006; Vestergaard, 2007). Diabetic osteopathy is a significant comorbidity of both forms of diabetes and is characterized by microarchitectural changes that decrease bone quality leading to increase risk of bone fracture in both types of diabetes (Lane et al., 2006; Hofbauer et al., 1999; Nyman et al., 2011; Thrailkill et al., 2005).

Limitations

This study needs further evaluation to confirm the mecahanism of action of KH through measurements of oxidative stress enzymes and the structural and cellular changes of the bones. Further evaluation of the expressions of the genes and protein related to bone formation and resorption may beconsidered in the future studies. To support the result of the BMD, bone mineral content should be measured which was not done in this study. This parameter will be done later on.

Conclusion

KH at the dose of 200 mg/kg/day showed potential protective agent against osteoporosis induced by long term glucocorticoid treatment. The protective effects could be attributed by the antioxidative and ant-inflammatory properties of KH. More extensive studies need to be done to explore the mechanisms of the protective effects of KH on osteoporosis induced by chronic glucorticoid administration.

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Availability of data and materials

All the data is available with the corresponding author.

Authors' contributions

All the authors had contributed in performing this study. Elvy Suhana Mohd Ramli drafted the proposal as well as the manuscript. Ima Nirwana Soelaiman, Norazlina Mohamad, Fairus Ahmad and Nur Azlina Mohd Fahami had edited the proposal and the manuscript. Amardev Singh and Mohd Amir Kamaruzzaman performed the animal treatment, laboratory work and statistical analysis. All the authors denied any conflict of interest in this study and manuscript.

Ethics approval and consent to participate

This research had obtained ethical approval from the Universiti Kebangsaan Animal Ethics Committee (UKMAEC) with the number: ANAT/PP/2016/ELVY/28-SEPT./789-OCT-2016-SEPT.-2018.

Competing interests

All the authors denied any competing interest in this study and manuscript.

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