COMBINED EFFECTS OF A CIRCUIT TRAINING PROGRAMME AND HONEY SUPPLEMENTATION ON BONE METABOLISM MARKERS IN YOUNG MALES

By

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Dissertation submitted in partial fulfillment
of the requirements for the degree
of Bachelor of Health Sciences (Exercise and Sports Science)

MAY 2011

ACKNOWLEDGEMENTS

First of the most, the greatest thank is to God the Almighty for giving me patience, strength, abilities and ideas for completing my final year project.

The greatest gratitude goes to my supervisor Dr. Ooi Foong Kiew for her assistance, guidance, and continuous advices in planning and execution of the present final year project. Great appreciation also goes to Mdm. Malisa Yoong bt Abdullah for her laboratory assistance. I would also like to acknowledgement all the staffs of the Exercise and Sport Science Programme and Sport Science Unit, Universiti Sains Malaysia, especially, Mdm. Jamaayah bt Meor Osman, Mdm Mazra bt Othman, Mr. Hafizi for their assistance throughout my study. My gratitude also goes to a USM Research University (RU) Grant for the financial support. A special thank to USM students for being the subjects in my final year project.

Special gratitude to my parents and family members who always support me in everything I do, they are the backbone of mine. I would also like to express my gratitude to lecturers for their recommendation and suggestion to my final year project. My gratitude also expends to all my friends for their help and moral supports.

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KESAN GABUNGAN PROGRAM LATIHAN LITAR DAN PENGAMBILAN MADU KE ATAS METABOLISMA TULANG BAGI LELAKI MUDA

ABSTRAK

PENGENALAN: Selain daripada aktiviti fizikal, kesihatan tulang boleh dipertingkatkan dan dikekalkan melalui pengambilan nutrisi yang mencukupi untuk pencegahan osteoporosis.Setakat ini, pengetahuan tentang kesan gabungan program latihan litar dan madu ke atas tulang di kalangan lelaki muda masih terhad. **MATLAMAT**: Kajian ini dijalankan untuk mengkaji kesan gabungan latihan litar dan pengambilan madu ke atas metabolism tulang selama 6 minggu bagi lelaki muda. **KAEDAH**: Empat puluh subjek lelaki muda (Umur: 19-25 tahun) telah dibahagikan kepada empat kumpulan yang terdiri daripada 10 orang dalam setiap kumpulan (n=10): kumpulan kawalan yang tidak bersenam dan tidak mengambil madu (C), kumpulan yang mengambil madu tanpa senaman (H), kumpulan yang bersenam tanpa mengambil madu (Ex) , kumpulan yang bersenam dan mengambil madu (HEx). Latihan litar ini terdiri daripada 1 jam/sesi, 3 kali seminggu selama 6 minggu. Subjek dalam kumpulan H dan HEx mengambil minuman madu sebanyak 300mL yang mengandungi 20g Madu Tualang 7 hari seminggu. Subjek dalam kumpulan HEx mengambil minuman madu 30 minit sebelum melakukan latihan litar.Sebaik sahaja sebelum dan selepas 6 minggu tempoh kajian, parameter-parameter antropometri subjek diukur dan sampel darah diambil untuk penentuan kepekatan serum alkali fosfatas (ALP) dan serum osteocalcin sebagai penanda-penanda pembentukan tulang, dan serum C-terminal telopeptide of type 1 collagen (1CTP) sebagai penanda penyerapan tulang. **KEPUTUSAN**: Pada pasca ujian, gabungan latihan litar dan pengambilan madu (HEx) didapati dapat meningkat secara signifikan ($p<0.05$) serum alkali fosfatas (penanda pembentukan tulang). Manakala, penurunan yang signifikann($p<0.05$) bagi serum 1CTP (penanda penyerapan tulang) dapat dikesan dalam kedua-dua kumpulan H dan HEx pada pasca ujian dibandingkan dengan nilai pra ujian

masing-masing. **KESIMPULAN**: Hasil kajian ini mencadangkan bahawa gabungan di antara latihan litar dan pengambilan madu dapat membawa kesan baik yang lebih nyata ke atas kesihatan tulang secara keseluruhan jika dibandingkan dengan latihan litar atau pengambilan madu sahaja di kalangan subjek lelaki muda.

COMBINED EFFECTS OF A CIRCUIT TRAINING PROGRAMME AND HONEY SUPPLEMENTATION ON BONE METABOLISM MARKERS IN YOUNG MALES

ABSTRACT

INTRODUCTION: Besides physical activity, bone health can be enhanced and maintained through adequate nutritional intake in prevention of osteoporosis. To date, little is known about the combination effects of a circuit training programme and honey supplementation on bone health in young males. **PURPOSE**: This study was carried out to investigate the effects of 6 weeks combined circuit training and honey supplementation on bone metabolism markers in young males. **METHODS**: Forty young male subjects (Age: 19 to 25 years old) were assigned into four groups, with ten subjects per group (n=10): sedentary without honey supplementation control (C), sedentary with honey supplementation (H), circuit training without honey supplementation (Ex), circuit training with honey supplementation (HEx) groups. Circuit training consisted of one hour per session, three times per week for six weeks. Subjects in H and HEx consumed 300mL of Tualang honey drink containing 20g of honey for 7 days per week. Subjects in HEx consumed honey drink 30 minutes before performing circuit training. Immediately before and after six weeks of experimental period, subject's anthropometry parameters were measured and blood samples were taken in order to measure concentrations of serum alkaline phosphatase (ALP), serum osteocalcin as bone formation marker, and serum C-terminal telopeptide of type 1 collagen (1CTP) as bone resorption marker. **RESULTS**: At post test, the combination of circuit training and honey supplementation (HEx) was found significantly ($p<0.05$) increased serum alkaline phosphatase (bone formation). Meanwhile, significant ($p<0.05$) reduction in serum 1CTP (bone resorption) was found in both H group and HEx group at post test compared to their pre test value respectively. **CONCLUSION**: The results of present study suggests that combination of circuit training and honey supplementation elicit more beneficial effects on

bone health generally compared to circuit training or honey supplementation alone in young males subjects.

CHAPTER 1

INTRODUCTION

Bone is an organ that gives form to the body, supporting its weight, protecting vital organs, and facilitating locomotion by providing attachments for muscles to act as levers. It also acts as a reservoir for ions, especially calcium and phosphate, the homeostasis of which is essential to life. The strength of a bone and its ability to perform physical functions depend on its structure and the intrinsic properties of the materials of which it is composed. The amount of bone (bone size, mass, and density), its spatial arrangement (shape, geometry, and microarchitecture), its composition (intrinsic properties of bone materials), and its turnover (rate and balance of formation and resorption) are all such determinants of its ability to perform mechanical functions and to resist fracture (Woolf & Akenson, 2008).

Osteoporosis which literally means porous bones, is a disease in which the bones become fragile and brittle. Throughout life, bone is very active and is constantly being "remodelled". During childhood, more bone is laid down than is removed. During early adulthood the two processes are balanced but bone continues to become thicker and stronger. Peak bone density, or peak bone mass is the maximum amount of bone in the skeleton during lifetime. After about the age of 40-50 years old, more bone is removed than is laid down, and very gradually the density of the bone begins to decrease, about 0.8% per year. During menopause, the decline in estrogen levels results in an accelerated bone loss of 3-5% per year (Heaney *et al.*,1978).

Honey is known to possess a variety of antioxidants and antibacterial substances that have been shown to inhibit growth of a wide range of bacteria and fungi. Honey can prevent fatigue and also used in beauty and personal care such as facial moisturizer and body scrub. Honey contains minerals such as calcium, phosphorus and magnesium which are believed to be vital for enhancing bone health.

A previous study on the effects of honey and its carbohydrate constituents i.e. glucose, fructose and raffinose on calcium absorption in rats in acute and chronic feeding reported that honey may have potential to boost calcium absorption and increase bone mineral density in rats (Ariefdjohan *et al,* 2008), implying that honey may elicit beneficial effects on bone.

Circuit weight training programs can be designed to increase muscular strength, endurance, power and cardio respiratory endurance (Gettman & Pallock, 1981). A circuit weight training program usually has 6-15 stations per circuit. The circuit can be repeated two to three times so that the total time of continuous exercise is 20-30 minutes. At each station, a load is selected that fatigues the muscle group in approximately 40%-55% of 1-RM). There is a 15-20 second rest period between exercise stations. Circuit weight training is usually performed 3 days a week for at least 6 weeks. In the present study, a circuit training programme consists of 2 circuits of 10 different exercises was carried out. The exercises include activities which are believed to be beneficial for increasing bone health in arms, legs and trunk of the subjects.

To date, no studies have been undertaken to determine the combined effects of a 6 weeks circuit training programme and honey supplemenation on bone metabolism markers in young males. Thus, the present study was proposed. A Malaysian local honey product 'Tualang' honey was consumed by the subjects, and the activities prescribed in the circuit training are believed to be beneficial for increasing bone health in arms, legs and trunk of the

subjects. Circuit training activities prescribed in the present study include rope skipping, elastic band leg exercise, split jump and burpee to increase bone strength of the legs; and push-ups, elastic band hand exercise, arm biceps and triceps dumb-bell exercises to enhance bone strength of the arms and back extension and sit-ups which aimed to improve bone strength in the trunk (Ooi, 2011).

1.1 OBJECTIVE OF THE STUDY

To determine the combination effects of 6 weeks circuit training and honey supplementation on bone metabolism markers such as serum alkaline phosphates, osteocalcin (bone formation markers), serum C-terminal telopeptide 1 collagen (1CTP) and serum total calcium in young males.

1.2 SIGNIFICANCE OF THE STUDY

It is hoped that results obtained from this study can be used for formulating guidelines in planning exercise and nutritional promotion for the maintenance of bone health in young males.

1.3 HYPOTHESIS

H_o: There are no significant differences in bone metabolism markers in combined circuit training and honey supplementation group compared to sedentary without supplementation control, honey alone and circuit training alone groups.

H_A: There are significant differences in bone metabolism markers in combined circuit training and honey supplementation group compared to sedentary without supplementation control, honey alone and circuit training alone groups.

1.4 OPERATIONAL DEFINITIONS

Circuit training programme: It consists of circuit training sessions with one hour per session, 3 times per week for a total of six weeks.

Bone metabolism: Measurements of blood parameters, i.e serum alkaline phosphates (ALP) and osteocalcin as bone formation markers, serum C-terminal telopeptide type 1 collagen (1CTP) as bone resorption marker, and serum total calcium.

Honey supplementation: Malaysian local honey (Tualang Honey) was consumed at a dosage of 20g per day, which was diluted with 300mL of plain water, seven days per week for six weeks by the subjects.

Young male subjects: A group of Malaysian males between 19-25 years old were recruited.

CHAPTER 2

LITERATURE REVIEW

2.1 ANATOMY AND PHYSIOLOGY OF BONE

2.1.1 Bone anatomy, histology and structure

Individual bones are classified according to their shape as long, short, flat or irregular. The fundamental constituents of bone are the bone cells and the extracellular matrix. By weight, mature bone matrix normally has approximately 35% organic and 65% inorganic material. The organic material consists primarily of a calcium phosphate crystal called hyroxyapatite.

The dense outer surface or cortex is composed of compact bone, and the centre or medulla is braced by narrow plates or trabeculae, a construction which gives maximum strength for minimum weight. The proportions of cortical and trabeculae bone differ at different sites in the skeleton. Trabeculae bone is predominant in the vertebrae and femoral head, but cortical bone predominates at the distal radius and femoral neck. The intertrochanteric area of the femur is 50% cortical and 50% trabeculae bone. This distribution and differential loss of cortical and trabecular bone in different scenarios accounts in part for the occurrence of different fractures in different situations: cortical bone loss predisposes to peripheral fractures such as of the hip and wrist, whereas trabeculaer loss predisposes to vertebral fractures (Woolf & Akesson, 2008).

2.1.2 Bone cells

Bone cells are categorised as osteoblasts, osteocytes and osteoclasts, which have different functions and origins. Osteoblasts produce new bone matrix. They have an extensive endoplasmic reticulum, numerous ribosome, and Golgi apparatuses. Osteoblasts produce collagen and proteoglycans, which are packaged into vesicles by the Golgi apparatus and released from the cell by exocytosis. Bone matrix produced by the osteoblasts covers the older bone surface and surrounds the osteoblasts cell bodies and processes (Philip, 2009).

Osteocytes are mature bone cells that maintain the bone matrix. Once an osteoblast becomes surrounded by bone matrix, it becomes osteocytes. The spaces occupied by the osteocytes cell bodies are called lacunae and the spaces occupied by the osteocytes cell processes are called canaliculi. Bone differs from cartilage in that the processes of bone cells are in contact with another through the canaliculi. Instead of diffusing through the mineralised matrix, nutrient and gases can pass through the small amount of fluid surrounding the cells in the canaliculi and lacunae, or pass from cell to cell through the gap junctions connecting the cell processes.

Osteoclasts are responsible for the resorption, or breakdown of bone. They are large cells with several nuclei. Osteoclasts release H^+, which produce an acid environmental necessary for the decalcification of the bone matrix. The osteoclasts also release enzymes that digest the protein components of the matrix. Through the process of endocytosis, some of the breakdown products of bone resorption are taken into the osteoclaste (Philip, 2009).

2.1.3 Bone remodelling

Bone undergoes a continuous process of resorption and subsequent formation known as remodeling. There are three primary factors that influence remodeling: (1) hormonal status, (2) weight-bearing physical activity and (3) dietary intake particularly calcium. The normal remodelling sequence takes 100-200 days. The sequence is initial activation of osteoclast precursors followed by osteoclastic bone resorption. There is then a reversal, with subsequent osteoblastic bone formation to repair the defect. This is followed by a resting phase before the cycle begins again (Nichols *et al.*, 2007).

Remodelling is responsible for the formation and resorption of bone. During remodeling, osteoclasts resorb damaged or fatigued bone, thus, making way for new bone, which is subsequently laid down by osteoblasts. Bone loss can occur when the work of the osteoclasts exceeds that of the osteoblasts. On the other hand, when osteoblast production is greater than resorption by osteoclasts, a net gain of bone is observed. Bone remodelling is a slow process, and it has been estimated that it takes 10 years to renew the entire skeleton (Ondrak & Morgan, 2007). The balance between bone resorption and bone formation is maintained through a complex regulatory system of systemic and local factors acting on bone cells, such as calcium regulating factors, sex hormones, growth factors, and cytokine.

2.1.4 OSTEOPOROSIS

Osteoporosis is a common metabolic bone disease. In other words, osteoporosis is a systemic skeletal disease, with the characteristic of bone loss and deteriorated of bone tissue micro-architecture, results in increase of fragility and the risk of falls or fractures, e.g. hip fracture (Zehnacker & Ougherty, 2007). Bone homeostasis depends on equilibrium of biochemical and mechanical environment in human body. Bone is continually being broken down by osteoclasts and formed by osteoblasts throughout life. When the rate of resorption exceeds that of formation, bone loss will happen. In addition, a high rate of bone turnover might be associated with an increased rate of bone loss in postmenopausal women, which appears to have negative influence on bone mineral density and increase fracture risk (Klentrou *et al.*, 2007). Osteoporosis is typically localised in the bulbar expansions of the long tubular bones, adjacent to the joints, and in the vertebrae the energy needed to break bone is low (Komadina, 2008).

Male osteoporosis is becoming increasingly recognised as a significant medical issue affecting the older male population. By the age of 90 years, one of every six men may have a hip fracture. Although there is an increase in morbidity and mortality in both in male and female after hip fracture, the mortality rate in men is higher (Cooper *et al.*, 1993). Osteoporosis in men is often a heterogeneous disorder and more than one factor contributes to this disease (Pande, 2001). Although most studies have been conducted on women, there is evidence to suggest genetic factors and positive family history of fractures are also important determinants of osteoporosis in men.

2.2 HONEY AND BONE HEALTH

Honey is a natural product with very complex chemical position. It is composed primarily of fructose and glucose but also contains 4 to 5 % fructooligosaccharides which serve as prebiotic agents. It contains more than 180 substances, including amino acids, vitamins such as vitamin D and K, minerals such as calcium, phosphorous and magnesium, and enzymes such as glucose oxidase and diastase (Al-Waili., 2003). Honey has been used as a food preservation agent (Lusby *et al.*, 2005).Besides that, flavanoids also presents in honey, which are categorised into three classes with similar structure: flavonols, flavones and flavanones. They contribute significantly to honey colour, taste and flavor, and have beneficial effects on health (Estevinho *et al.*, 2008).

In the present study, Tualang honey was used. Tualang honey is a wild multi-floral honey produced by *Apis dorsata*. The honey obtained its name from a tall *Koompasia excelsa* tree known locally as "Tualang tree", where the bees built their hives high on the trees to avoid disturbances by human and animals. It also has antibacterial properties similar to manuka honey (Tan *et al.*, 2009) with good anti-oxidant properties (Mahaneem *et al.*, 2010).

A previous study (Lily, 2010) which investigated effects of Tualang honey on postmenopausal women suggested that daily intake of honey at 20 mg/day for four month was found to be safe to be used and have the same effect on bone densitometry when compared with hormone replacement therapy. This study showed that honey may be beneficial for bone health in postmenopausal women.

2.3 EFFECTS OF EXERCISE ON BONE HEALTH

Among all the types of exercise, weight bearing exercise are believed to be most beneficial for eliciting bone health (Ooi, 2011). Examples of weight bearing exercise are walking, jogging, running, dancing and resistance exercise. Exercise stimulates tissue growth in muscle and bone, which benefits both the young and the old. In general, regular exercise slows the loss of muscle mass, strenghtens bones and reduces joint and muscle pain. In addition, mobility and balance can be improved with exercise, which reduces the risk of falling and serious injury such as a hip fracture (Campbell *et al.*,1997).

Turner *et al* (1998) reported that physical activity has been shown to have positive impacts on bone density. The adaptations in aging skeletal muscle to training exercise enhance the ease of carrying out the activities of daily living and exert a beneficial on osteoporosis. Muscle stresses are important in fostering and maintaining bone mineral density, therefore, moderate levels of physical activities which can increase muscular strength exert a protective effect against hip fracture in postmenopausal women.

In younger population, exercises have been reported to be beneficial in increasing bone health (Ondrak & Morgan, 2007). The type, frequency, and duration of exercise all influence study findings. The positive effect of exercise and physical activity, especially weight bearing, on maximising peak bone mass during the growing years has been reported (Nichols *et al.*, 2007). In the study of Janz *et al*, (2001) it was reported that there were higher spine, hip and total body bone mineral content, and total body bone mineral density in 4 years old boys compared to 4 years old girls due to higher involvement in physical activities in boys. Physical activity and calcium intake are important to the development of bone mineral density and bone mineral content during the prepubertal years (Ondrak & Morgan, 2007).

Regarding beneficial effects of resistance training, resistance training is any exercise that causes the muscles to contract against an external resistance with the expectation of

increases in muscular strength, tone, mass and endurance. The effect of resistance training to the muscle and bone growth and strength support the principle of overload that state muscle and body cell increase in strength by being force to contact near maximum. Elastic band is one type of resistive exercises as an exercise intervention to improve the muscle strength among the participant (Shirazi *et al*, 2007). Circuit weight training programs can be designed to increase muscular strength, endurance, power and cardio respiratory endurance (Gettman & Pallock, 1981). A circuit weight training program usually has 6-15 stations per circuit. The circuit can be repeated two to three times so that the total time of continuous exercise is 20-30 minutes. At each station, a load is selected that fatigues the muscle group in approximately 40%-55% of 1-RM). There is a 15-20 second rest period between exercise stations. Circuit weight training is usually performed 3 days a week for at least 6 weeks. In the present study, a circuit training programme consists of 2 circuits of 10 different exercises was carried out. The exercises include activities which are believed to be beneficial for increasing bone health in arms, legs and trunk of the subjects. The circuit training activities include rope skipping, elastic band leg exercise, split jump and burpee to increase bone strength of the legs; and push-ups, elastic band hand exercise, arm biceps and triceps dumb-bell exercises to enhance bone strength of the arms, and back extension and sit-ups which may improve bone strength in the trunk (Ooi, 2011).

2.4 COMBINED EFFECTS OF HONEY AND EXERCISE ON BONE HEALTH

Jumping exercise is a type of weight–bearing exercise that causes muscles and bones to work against gravity. Such exercises exert a loading impact and stretch and contract the muscles, which stimulate bone to strengthen itself. In a previous study, Ooi *et al* (2010) reported that after 8 weeks of experimental period, tibial and femoral maximal load were significantly increased in combined jumping exercise and honey supplementation group in the

rats. These findings were along with significantly reduction in serum 1CTP (bone resorption marker) in jumping and honey supplementation group. This study findings imply that there are beneficial effects on tibia and femur bone strength and reduction in bone resorption marker, with combined jumping exercise and honey supplementation.

The effects of another form of exercise i.e aerobic dance on bone have also been investigated. It was reported by Noorsuzanawati (2010) that, there were significantly in increased serum alkaline phosphatase and reduction in serum 1CTP in combined aerobic exercise and honey group when compared to control group. The present study suggests that combination of aerobic dance exercise and honey supplementation may elicit beneficial effects on bone health generally. Since the combined effects of a circuit training programme and honey spplementation on bone metabolsim markers have not been carried out, thus, the present study was proposed.

CHAPTER 3

MATERIALS AND METHODS

3.1 Subjects

Forty young Malaysian male subjects with age ranging from 19 to 25 years old were recruited in this study. The inclusion criteria of the subjects including the subjects have to be free any health problems and they did not have the habit of taking honey as daily supplementation prior to the experiment. The subjects were assigned into four groups, with each group consisting of ten subjects. The subjects were assigned randomly into the control and experimental groups. Each subject was given a detail explanation about the objectives, procedures, benefits, risks and possible discomforts experienced in this study. Ethical Approval (Appendix B) and subjects' information and consent forms (Appendix C) approved by the Universiti Sains Malaysia Research and Ethical Committee were distributed to all the subjects. Subjects were reminded regarding their participation in this study as being voluntary and they were permitted to stop being a part of this study at any time during the course of the study period.

3.2 EXPERIMENTAL DESIGN

3.2.1 Subjects grouping

In the present study, subjects were assigned into four groups, with ten subjects per group (n=10) : six weeks of sedentary without honey supplementation control (C), six weeks of sedentary with honey supplementation (H), six weeks of circuit training without honey supplementation (Ex), six weeks of circuit training with honey supplementation (HEx) groups (Figure 3.1).

3.2.2 Blood sample taking and anthropometric measurement

Immediately before six weeks of experimental period, all the subjects were required to have the blood sample taking and anthropometry measurement sessions in the Exercise and Sport Science Laboratory, School of Health Sciences, Health Campus, Universiti Sains Malaysia. Blood taking was carried out again after 6 weeks of experimental period.

In each blood taking, 3 mL of blood was taken from each subject. The blood sample was centrifuged for 15 minutes of 3000 RPM at 4° C (Health-Rotino 46RS,Germany), the serum obtained was then divided into equal portions and stored at -80° C (Heto Ultra Freezer 3410, Denmark) until subsequent analysis of serum alkaline phosphatase, osteocalcin, C-terminal telopeptide of type 1 collagen (1CTP), and total calcium.

After the blood taking, subjects physical and physiological measurements such as height, weight, and percentage of body fat were carried out on the same day. The subjects body heights were measured by stadiometer (Seca 220, Germany), the body weight and percentage body fat were measured by a digital bioelectric impedance analysis device.

FLOW CHART OF RESEARCH PROCEDURE

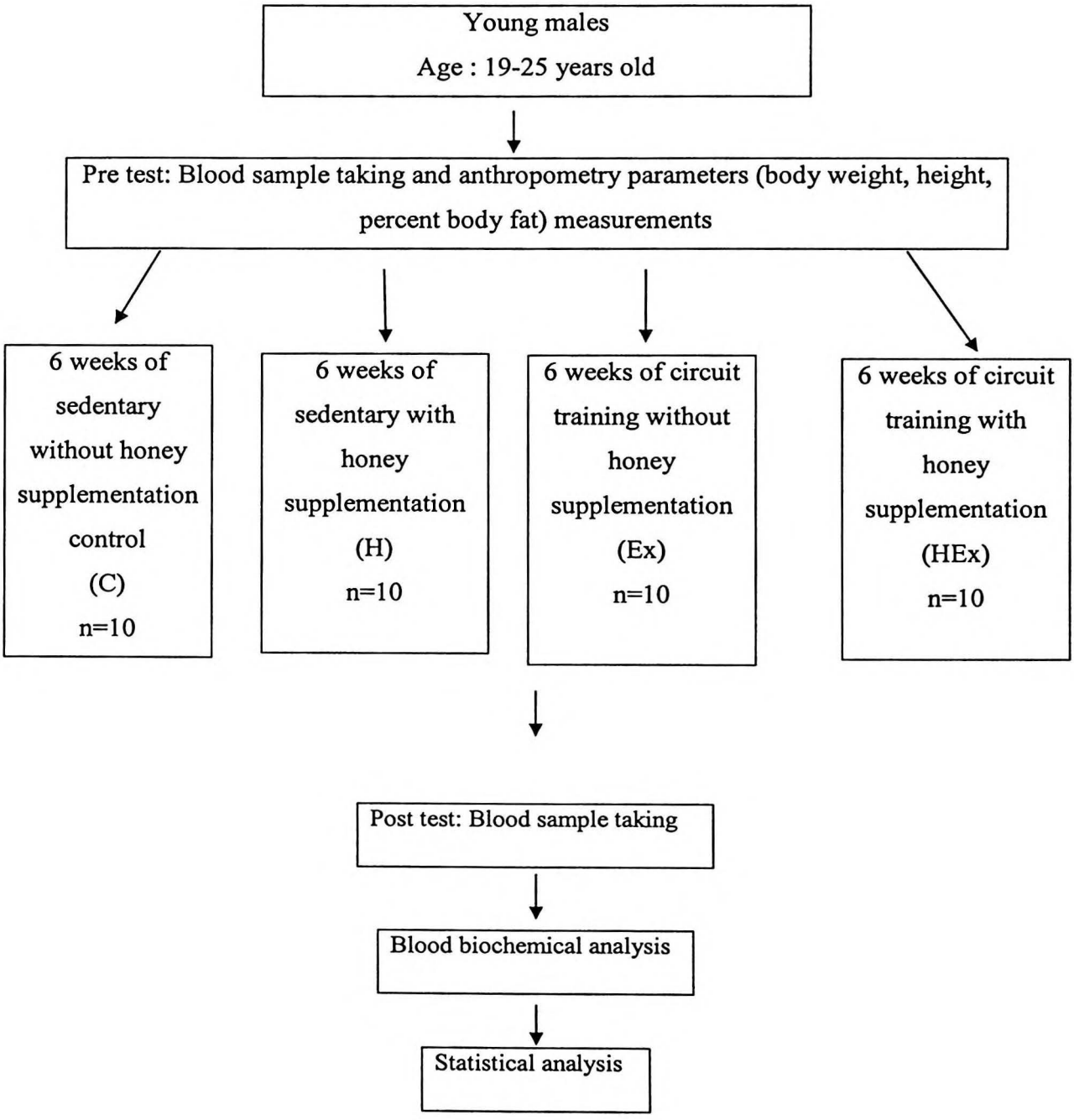

Figure 3.1 Flow chart of the experimental design

3.2.3 Circuit training programme

The subjects in both the exercise without supplementation group (Ex) and honey supplementation with exercise group (HEx) were required to carry out circuit training sessions, one hour per session (5.30 p.m to 6.30 p.m), three times per week for six weeks. The sessions started with 10 minutes of warm-up and ended with 5 minutes of cooling down activities.

In the present study, the circuit training programme consists of two circuits. In each circuit, subjects performed 10 different exercises in 10 different stations (one type of exercise per station, each subject spent 30 seconds in one particular station). The work rest ratio was 1:2, where subjects exercised for 30 seconds for one activity, and rested for one minute before continued with the next activities. Resting time between circuits was five minutes.

Types of activities precsribed in this circuit training programme were hand elastic band, leg elastic band, free weight dumbell triceps extension, rope skipping, free weight dumbell concentration curl, sit up, back extension, burpee, push up and split squat. It is beleived that rope skipping, elastic band leg exercise, split jump and burpee can increase bone strength of the legs; and push-ups, elastic band hand exercise, arm biceps and triceps dumb-bell exercises can enhance bone strength of the arms. Exercises such as back extension and sit-ups can improve bone strength in the trunk (Ooi, 2011).

3.2.4 Honey Supplementation

Three hundred ml of honey drink which containing 20g of Tualang honey was consumed by the subjects of hiney supplementation group (H) and honey supplementation with exercise group (HEx) per day, seven days per week for six weeks. Subjects in HEx consumed 300 mL of honey drink 30 minutes before performing circuit training. The mixture

of honey was prepared by mixing 20g of honey with 300ml of plain water. In the present study, a Malaysian local product, i.e, 'Tualang' honey was used.

3.2.5 Blood Biochemical Analysis

3.2.5.1 Analysis of serum alkaline phosphatase

Serum alkaline phosphatase (ALP) was analysed using an automatic analyser (Hitachi Automatic Analyser 912,Bohringer Mannhein, Germany) with commercially available reagent kits (Bm/Hitachi 902, Germany). Alkaline phosphatase are plasma membrane enzyme which include several isoenzymes: placental, intestinal, germ cell, kidney, liver, and bone. Over 90% of the total ALP measured in serum is derived from liver and bone. Basically, alkaline phosphatase is determined using an optimised p-nitrophenyl phosphate concentration and 2-amino-2methyl-1-propanol in the presence of cations magnesium and zinc as buffers. When a sample of serum is mixed with a buffer containing 2-amino-2methyl-1-propanol, magnesium acetate, zinc sulfate, and then p-nitrophenyl phosphate, p-nitrophenyl phosphate is cleaved by alkaline phosphatase into phosphate and p-nitrophenol.

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow{\text{Alkaline phosphatase}} \text{phosphate} + \text{p-nitrophenol}$$

The p-nitrophenol, a yellowish end product, is proportional to the alkaline phosphatase concentration which is then measured photometrically (Hitachi Automatic Analyser 912, Bohringer Mannheim, Germany).

3.2.5.2 Analysis for serum osteocalcin

Serum osteocalcin was analysed using a commercially available enzyme-linked immunosorbent assay kit (Nordic Bioscience Diagnostics RatMID^{TM} Osteocalcin ELISA, Denmark) and the concentration was determined using a photometric microplate reader (Molecular Devices; $Versa_{max}$ tunable microplate reader, U.S.A).

Serum osteocalcin concentration determination is based on the competitive binding of an enzyme-linked monoclonal antibody to soluble osteocalcin in the serum sample or to immobilised osteocalcin. In the assay, biotinylated synthetic human osteocalcin is immobilised by binding to a streptavian-coated microtitre wells. When a solution of enzyme-linked monoclonal antibody is added to the wells together with the serum samples, the soluble osteocalcin in the serum competes with the monoclonal antibody for the immobilised osteocalcin. A solution of peroxidase enzyme conjugate is then added and binds determined by the addition of a choromogenic enzyme substrate (tetramethylbenzedine). The coloured end product of the reaction between enzyme and substrate is finally determined spectrophotometrically, where the absorbance level is inversely proportional to the concentration of osteocalcin in the serum sample.

Procedure of the test

Frozen serum samples were first thawed for half an hour and then vortex-mixed (Thermolynen 37600, U.S.A) for 30 seconds. A volume of 100 µl of biotinylated osteocalcin was pipetted into each well, which was pre-coated with streptavidin. The wells were then covered with sealing tape and incubated for 30 minutes at room temperature on a microtitre plate mixing apparatus (IKA Vibrax VXR basic, U.S.A) which was shaken at 300 rpm. After that, the microwells were washed 5 times with washing solution by using an automated plate

washer. Next, a solution of monoclonal antibody was mixed with the incubation buffer at a volumetric ratio of 1:1000. Subsequently, 20 µl of standards, control, and serum samples were pipetted into their respective wells, followed by 150 µl of the mixture of monoclonal antibody solution and incubation buffer. The wells were then covered with sealing tape and incubated for 60 minutes at room temperature on the microtitre plate mixing apparatus which was shaken at 300 rpm. After that, the microwells were washed 5 times with washing solution using an automated plate washer. After washing, a volume of 100 µl of peroxidase enzyme conjugate was pipetted to each well, the wells were then covered with sealing tape, and incubated for another 30 minutes at room temperature on the microtitre plate mixing apparatus which was shaken at 300 rpm. Following this, the microwells were again washed 5 times with the washing solution using an automated plate washer. After three washings, 100 µl of tetramethylbenzedine (TBM) chromogenic substrate solution was pipetted into each well. The wells were then covered with a sealing tape and incubated for 15 minutes in darkness on a microtitre plate mixing apparatus which was shaken at 300 rpm. Subsequently, a volume of 100 µl of sulfuric acid was pipetted into each well to stop the colour reaction. The absorbance of the solution in the wells was then measured within 2 hours on a microplate reader (Molecular Devices; $Versa_{max}$ tunable reader, U.S.A) at a wavelength of 450 nm. Osteocalcin concentration of each sample was calculated automatically by the ELISA microlate reader system.

3.2.5.3 Analysis of serum C-terminal telopeptide of type 1 collagen (1CTP)

Serum C-terminal telopeptide of type 1 collagen (1CTP) was analysed using a commercially available enzymeimmunoassay kit (Orion Diagnostic UniQ 1CTP, EIA, Finland), and the concentration was determined by a photometric microplate reader (Molecular Devices; $Versa_{max}$ tunable microplate reader, U.S.A).

Serum C-terminal telopeptide of type 1 collagen (1CTP) determination is based on the competitive immunoassay technique. A known amount of peroxidase labelled 1CTP and an unknown amount of unlabelled 1CTP in the serum sample compete for the limited number of high affinity binding sites of the primary antibody. A secondary antibody is then directed against the primary antibody and binds the antibody-antigen complex, which enables separation of bound and free antigen. After washing away the free antigen, the amount of labelled 1CTP left in the well is inversely propotional to the amount of 1CTP in the serum sample. The amount of labelled 1CTP is measured by incubation with a substrate that produces a coloured end product. The absorbance of the coloured end product is measured specttrophotometrically, where the absorbance level is inversely proportional to the concentration of 1CTP in the serum sample.

Procedure of the test

Frozen serum samples were thawed for half and hour and vortex-mixed (Thermolyne 37600, U.S.A) for 30 seconds prior the analysis of 1CTP concentration. Fifty microlitre aliquots of standards, control, and serum samples were pippetted into appropriate microlitre wells and followed by 50 µl of 1CTP enzyme conjugate into all wells. Following that, 50 µl of 1CTP antiserum (secondary antibody) was pipetted into all wells within 3 minutes. The wells were then covered with sealing tape and incubated for 2 hours at room temperature on

the microtitre plate mixing apparatus which was shaken at a 300 rpm. After that, the microwells were washed 5 times with the washing solution using an automated plate washer. After washing, 100 µl of 1CTP substrate was pipetted into all wells. The wells were then covered with sealing tape and incubated for 30 seconds at room temperature on the microtitre plate mixing apparatus which was shaken at a rate of 300 rpm. Following that, 100 µl of sulfuric acid was pipetted into each well to stop the colour reaction. Absorbance was then measured within 10 minutes using a microplate reader (Molecular Devices; $Versa_{max}$ tunable microplate reader, U.S.A) at a wavelength of 450 nm. The 1CTP concentration of each sample was calculated automatically by the ELISA microplate reader system.

3.5.2.4 Analysis for serum total calcium

Serum total calcium was analysed colorimetrically (Hitachi Automatic Analyser 912, Bohringer Mannheim, Germany) using commercially available reagent kits (Roche Diagnostic GmbH, Germany).

Serum total calcium concentration determination is based on the reaction of calcium with o-cresolpthalein complexone in alkaline solution. When serum sample is mixed with ethanolamine buffer and subsequently with o-cresolpthalein complexone, a purple coloured calcium -o-cresolpthalein complex is formed.

$$\text{Calcium} + \text{o-cresolpthalein complexone} \xrightarrow{\text{Alkaline solution}} \text{calcium-o-cresolpthalein complex}$$

The colour intensity of the purple complex formed is directly proportional to the calcium concentration and the intensity of the purple complexs measured photometrically or colorimetrically.

3.2.6 Statistical Analysis

Statistical software in the Statistical Package for Social Sciences (SPSS) Version 18.0 was used for the statical analysis. All data are expressed as mean ± standard deviation (SD). Repeated measures ANOVA were performed to determine the significance of the difference between and within groups. Statistical significance was accepted at $p<0.05$

3.2.7 Calculation of sample size

Sample size of the present study was calculated by G power software. The power of the study was set at 80% confident interval. The calculated sample size was 9 subjects per group. However, it was estimated that the drop out rate due to stop of participation in the study by the subjects during experimental period would be 10%, therefore, the actual number of the subjects recruited in this study was 10 subjects per group.