EFFECTS OF BEE PROPOLIS SUPPLEMENTATION ON NEUTROPHILS COUNT FOLLOWING PROLONGED RUNNING

By

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In the name of Allah, the most Gracious and most merciful

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LIST OF ABBREVIATIONS

BMI	Body Mass Index
MNC	mononuclear cells
CAPE	Caffeic Acid Phenethyl Ester
IL	Interleukin
$km.h^{-1}$	kilometre per hour
kg	kilogram
km	kilometre
mg	milligram
min	minutes
mL	milliliter
RPE	Rating of Perceived Exertion
TNF	Tumour Necrosis Factor
URTI	Upper Respiratory Tract Infections
VO_2	Oxygen Uptake
VO_{2max}	Maximum Oxygen Uptake

ABSTRAK

KESAN SUPLEMEN PROPOLIS LEBAH TERHADAP BILANGAN NEUTROFIL BERIKUTAN LARIAN BERPANJANGAN

PENDAHULUAN: Kajian tentang keberkesanan suplemen propolis lebah untuk meningkatkan sistem ketahanan badan telah dijalankan. Walau bagaimanapun, setakat ini, hanya sedikit maklumat yang diperolehi mengenai keberkesanan suplemen propolis lebah terhadap sistem ketahanan badan dalam kalangan pelari rekreasi lelaki. **TUJUAN:** Untuk mengkaji kesan suplemen propolis lebah terhadap bilangan neutrofil dan beberapa parameter fisiologi berikutan larian berpanjangan dalam kalangan pelari rekreasi lelaki. **KAEDAH:** Sebelas peserta lelaki yang sihat (Umur: 21.0±1.5 tahun; BMI: 22.3±2.5 $kg.m^{-2}$) telah menyertai kajian ini. Peserta melakukan 3 ujian 'preliminary' yang melibatkan ujian pengambilan oksigen sub-maksimum, ujian pengambilan oksigen maksimum (VO_{2max}) dan ujian pembiasaan larian. Kemudian, peserta melakukan ujian larian pertama (berlari selama 90 minit pada 60% VO_{2max}) setelah berpuasa pada malam hari. Setelah itu, kajian ini diteruskan dengan pengambilan suplemen propolis lebah selama 4 minggu iaitu 2 pil setiap hari (500 mg propolis lebah dalam setiap pil). Selepas 4 minggu pengambilan suplemen propolis lebah, peserta melakukan ujian larian yang kedua yang mana protokolnya adalah sama dengan ujian larian pertama. Dalam setiap ujian larian, sampel darah diambil sebelum, semasa, selepas dan 1 jam selepas ujian larian dijalankan. Disamping itu, pengambilan oksigen (VO_2) dan kadar denyutan jantung diambil sebelum, semasa, dan selepas ujian larian dijalankan. **KEPUTUSAN:**

Kajian ini mendapati bahawa bilangan neutrofil telah meningkat dengan ketara semasa senaman dalam kedua-dua ujian larian. Selain itu, bilangan neutrofil tidak menunjukkan perbezaan yang ketara antara kedua-dua ujian. Begitu juga, VO_2 dan kadar denyutan jantung peserta tidak menunjukkan perbezaan yang ketara antara kedua-dua ujian. **KESIMPULAN:** Kesimpulannya, pengambilan suplemen propolis lebah tidak mempunyai kesan terhadap bilangan neutrofil, VO_2 dan kadar denyutan jantung berikutan larian yang berpanjangan dalam kalangan pelari rekreasi lelaki.

ABSTRACT

EFFECTS OF BEE PROPOLIS SUPPLEMENTATION ON NEUTROPHILS COUNT FOLLOWING PROLONGED RUNNING

INTRODUCTION: The effect of nutritional supplementation for improving immune system have been investigated. However, to date, little is known about the effectiveness of bee propolis supplementation on immune system in male recreational runners. **PURPOSE:** To investigate the effects of bee propolis supplementation on neutrophils count and selected physiological parameters (oxygen uptake and heart rate) following prolonged running in male recreational runners. **METHODS:** Eleven healthy male participants (Age: 21.0±1.5 years; BMI: 22.3±2.5 $kg.m^{-2}$) were recruited in this study. Participants performed 3 preliminary tests which include a submaximal test, a maximum oxygen uptake (VO_{2max}) test and a familiarisation test. After that, participants performed the first exercise trial (90 min running at 60% VO_{2max}) after an overnight fast. This was followed by 4 weeks consumption of bee propolis where they had to consume 2 tablets per day (500 mg of bee propolis per tablet). After the 4 weeks of consumption period, participants performed the second exercise trial which was carried out as similar as to the first exercise trial. During each trial, blood samples were collected pre-, during, post-, and 1 h post-exercise. In addition, oxygen uptake (VO_2) and heart rate (HR) were measured pre-, during, and post-exercise. **RESULTS**: This study discovered that neutrophils count was significantly increased during exercise in both trials. However, neutrophils count was not significantly different between both trials. Likewise, the

VO_2 and heart rate (HR) of the participants were not significantly different between both trials. **CONCLUSION:** As a conclusion, there were no significant effect of bee propolis supplementation on the neutrophils count, VO_2 and HR following prolonged exercise in male recreational runners.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The term immunity refers to all of the mechanisms used in the body to protect against foreign agents. The origin of this term is from the Latin term *immunitas* (meaning "freedom from") (Powers and Howley, 2012). Critical homeostatic system that recognises, attacks and destroys foreign agents in the body is known as immune system. Infection occurs due to constant attack from the foreign agents such as bacteria, viruses and fungi. That means, immune system plays an important role in maintaining homeostasis in the body in order to protect the body against infection.

To date, athletes are very concerned about the effects of exercise on immune function based on the cumulating evidences which show that prolonged exercise may suppress immune function and in turns may increase infection risk, mostly upper respiratory tract infection (URTI). These URTIs are commonly referred to as colds and are caused by more than 200 different viruses. Currently, URTIs are the most common types of infections worldwide and the average adult suffers from two to five colds a year (Powers and Howley, 2012).

Besides, Nieman (2001) also found that immune function suppressed dramatically after each bout of prolonged and intensive exercise. During this "open window" of altered immunity (which may last between three and 72 hours, depending on the immune measure), viruses and bacteria may gain a foothold,

increasing the risk of subclinical and clinical infection. Hence, it has been shown that infection may decrease athlete's performance.

Certain nutritional strategies have been shown to limit exercise-induced immune dysfunction and thus being recommended to athletes who are engaged in heavy training and competition. These nutritional strategies include ensuring adequate intake of macronutrients (carbohydrate, protein, and fat), avoidance of deficiencies of micronutrients (e.g. the minerals iron, zinc, copper and the vitamins A, B12, C, D, E, and folic acid), and taking supplements such as antioxidants, probiotics and herbs.

To our knowledge, bee propolis is a type of bee product which is reported as an immune booster. Propolis is a natural hive product collected from buds and produced by the honeybee. It has been long used in folk medicine. It has been proved on a scientific basis that is possesses versatile biological activities, including antimicrobial, anti-inflammatory, regenerative, and antioxidant (Ozturk *et al.*, 2000). It has been found that propolis has therapeutic effect on wound healing, ineffective wounds and inflammations of the skin and on other skin diseases (Ozturk *et al.*, 2000). Nevertheless, we found limited studies have been conducted to investigate the effects of bee propolis on athletes' immune function to date. Thus, the present study is warranted to fill in this gap of knowledge.

1.2 Objectives

To determine the effect of bee propolis supplementation (2 tablets per day for 4 weeks) on neutrophils count and selected physiological parameters (oxygen uptake and heart rate) following prolonged exercise (90 min running at 60% VO_2max) in runners.

1.3 Hypotheses

Ho_1 : There are no significant effect of bee propolis supplementation on physiological parameters (oxygen uptake and heart rate).

H_{A1} : There are significant effect of bee propolis supplementation on physiological parameters (oxygen uptake and heart rate).

Ho_2 : There is no significant effect of bee propolis supplementation on neutrophils count.

H_{A2} : There is significant effect of bee propolis supplementation on neutrophils count.

1.4 Significance of the study

Since to date, to our knowledge, there is limited study investigating the possible beneficial effects of bee propolis on immune function in athletes in response to exercise, it is hoped that finding from this study may add knowledge regarding nutritional strategy to limit exercise-induced immune depression among athletes. If the finding is positive, we may suggest athletes to consume this bee propolis as supplement.

CHAPTER 2

LITERATURE REVIEW

2.1 Bee propolis and its benefits

Bee propolis is a natural, non-toxic substance collected from bee's wax that has been used for many years in folk medicine. It is a resinous material collected by bees from various plant sources and mixed with the bee's salivary enzymes and beeswax. Bees use it to repair the hive walls and to protect the colony from disease (Tomazevic and Jazbec, 2013). Bee propolis is a complex natural matrix that honey bees collect from tree buds, saps and other plant sources. The most common European propolis is obtained from resins of Populus tree, and the Moroccan black propolis is from that of *Euphorbia resinifera* (Vit *et al.*, 2015).

Bee propolis contains amino acids, flavonoids, terpenes and cinnamic acid derivatives (Neiva *et al.*, 2014). In addition, bee propolis also contains Caffeic Acid Phenethyl Ester (CAPE), an active component of propolis extracts and has been demonstrated exhibited antioxidant properties (Ilhan *et al.*, 1999). Since bee propolis has been noted for multiple biological effects, it is widely used for the prevention and treatment of variety of diseases. Propolis and its extracts have long been used for the prevention and treatment of a variety of diseases and it has attracted much attention in recent years as a useful substance applied in medicine and cosmetics. This is because, a significant number of reports have described numeorus biological activities of propolis whih include antibacterial, anti-inflammatory,

immunomodulatory, antioxidant, anticancer, hepatoprotective and many other (Tyszka-Czochara *et al.*, 2014).

2.2 Bee propolis and immune function

Despite numerous *in vitro* and animal studies, to date, to our knowledge, there was no human study investigated the effects of bee propolis on immune function, especially on immune cells count. Previously, the ethanol extract of Brazilian propolis has been shown to exhibit anti-influenza virus activity *in vitro* and *in vivo* and ameliorate influenza symptoms in mice (Takeshita *et al.*, 2013). The researchers found that bee propolis ameliorated pneumonia in infected mice by limiting excess cellular immune responses.

In another animal study, mice were divided into three experimental groups: group 1 consisted of control non-diabetic mice that received daily supplementation of 100 µL of distilled 70 % ethanol via oral gavage for one month; group 2 consisted of diabetic mice that received 100 µL of 70 % ethanol daily via oral gavage for one month; and group 3 consisted of diabetic mice that were supplemented daily with the ethanol-soluble derivative of propolis (100 mg/kg body weight) dissolved in 100 µL of 70 % ethanol via oral gavage for one month (Al Ghamdi *et al.*, 2015). Therefore, the volume of daily supplementation received by each mouse in the three groups was constant and did not exceed 100 µL. It was found that the oral supplementation of diabetic mice with the ethanol-soluble derivative of propolis significantly increased the circulating lymphocytes count compared to diabetes induction alone.

In a separate study, researchers investigated the chemical composition and the biological properties of propolis (Campos *et al.*, 2015). The antimicrobial activity

of propolis was accessed in gram-positive and gram-negative bacteria. It was noted that propolis was active against all microorganisms and showed antioxidant activity by scavenging free radicals, inhibiting haemolysis and lipid peroxidation in human erythrocytes incubated with an oxidising agent. These results showed that propolis has important therapeutic activities, suggesting its potential application in the pharmaceutical industry, as well as in health foods, beverages and nutritional supplements.

2.3 Immune system

The immune system is a critical homeostatic system that recognises, attacks and destroys foreign agents in the body. Obviously, this is important because the body is constantly under attack from foreign agents such as bacteria, viruses and fungi that promote infection (Gleeson, 2006). By protecting the body against infection, the immune system plays an important role in maintaining homeostasis in the body. The immune system is a network of cells, tissues and organs that work together to defend the body against attacks by "foreign" invaders. These are primarily microbes, tiny organisms such as bacteria, parasites and fungi which can cause infections. Viruses also cause infections, but are too primitive to be classified as living organisms (Powers and Howley, 2012).

The immune system is amazingly complex. It can recognise and remember millions of different enemies, and it can produce secretions and cells to match up with and wipe out nearly all of them (Gleeson, 2006). The immune response can be divided into innate immunity and adaptive immunity which works synergistically. Innate immunity is the first response to physical or chemical foreign agents and it

occurs naturally and immediately, providing the first line of defence in early stages of the infection. The innate immunity is comprised of phagocyte cells (e.g. neutrophils, monocytes, eosinophils), natural killer cells, soluble factors (e.g. complement, cytokines, lysozymes, and acute phase proteins), as well as the mucosal immune responses. The innate mucosal defences are the first line of defence against pathogens present at the mucosal surfaces (Wolach, 2012). The adaptive immunity involves the antigen-antibody response. It includes B and T lymphocytes and the immunoglobulins. In the innate immunity phagocytes can recognise and act immediately against the foreign agent without prior exposure, while the adaptive immunity is characterized by a specific response to the infectious agent, becoming fully activated after a lag period.

2.3.1 Neutrophils

Neutrophils are well recognised as one of the major players during acute inflammation. It is a type of white blood cells (leukocytes) and a granulocyte. They are typically the first leukocytes to be recruited to an inflammatory site and are capable of eliminating pathogens by multiple mechanisms. Neutrophils help fight infection by ingesting microorganism and releasing enzymes that kill the microorganisms. Neutrophils are leukocytes that also participate as phagocytes during a bacterial invasion. Neutrophils have been called professional killers that are "on call" in the blood. Once neutrophils are summoned to the infected area, they migrate through the blood vessels, then through interstitial tisue, in a process called chemotaxis. Then, it becomes activated to begin destroying the foreign. Neutrophils also produce cytokines that can alert other immune cells. Further, activated

neutrophils also release chemicals that promote increased blood flow to the infected area.

Neutrophils functions extend beyond their roles in acute infection, and these findings are altering our understanding of the life and death of the neutrophils. Neutrophils were always considered short-lived cells with a half-life in the circulation of approximately 1.5 and 8 hours in mice and humans, respectively. Neutrophils are continuously generated in the bone marrow from myeloid precursors. Their daily production can reach up to 2×10^{11} cells. During inflammation the number of neutrophils in tissues increases, and with time the cells die and are removed by macrophages and dendritic cells. The process of neutrophils maturation is under the control of transcription factors.

2.4 Factors affecting immune function

Exercise and nutrition are among those factors that affect immune function. Numerous studies have been conducted regarding the effect of various types of exercise on immune function and infection risk. In addition, numerous researches have shown that certain types of supplements may help to reduce exercise-induced suppressed immune function.

2.4.1 Exercise and immune function

It has been shown that regular bouts of moderate aerobic exercise catch fewer colds than both sedentary individuals and people who engage in high intensity or long-duration exercise. For instance, both epidemiological and randomized studies consistently report that regular exercise results in an 18–67% reduction in the risk of upper respiratory tract infection (URTI) (Mackinnon, 2000). Interestingly, this

exercise-induced protection against infection can be achieved with many types of aerobic activity, for instance, walking, jogging, cycling, swimming, sports play and aerobic dance. In general, it appears that 20–40 minutes of moderate intensity exercise (~ 40–60% of VO_{2max}) is adequate to promote a beneficial effect on the immune system (Mackinnon, 2000). Importantly, this exercise induced reduction in the risk of URTI occurs in both men and women of all ages.

Each bout of moderate aerobic exercise causes an increase in blood levels of natural killer cells, neutrophils, and antibodies. Therefore, an acute bout of exercise provides a positive boost to both the innate immune system which are natural killer cells and neutrophils and the acquired immune system which is antibodies. Note that this exercise induced boost in immune function is transient and that the immune system returns to pre-exercise levels within 3 hours (Mackinnon, 2000). In addition, people who engage in regular exercise may also benefit from an improved psychological well-being, in example, less emotional stress, good nutritional status and a healthy lifestyle, such as adequate sleep. Each of these factors has been linked to a reduced risk of infection and therefore may also be a connection between regular exercise and lowered risk for URTI.

On the other hand, an endurance athletes experience a high incidence of URTI during intense training and after major competition. A single bout of acute intense exercise may suppress immune parameters for several hours afterwards. There is growing evidence supported that, for several hours subsequent to heavy exertion, several components of both the innate (e.g., natural killer cell activity and neutrophils oxidative burst activity) and adaptive (e.g., T and B cell function) immune system exhibit suppressed function (Nieman, 1997). For example, three

consecutive days of high-intensity exercise (85% of VO_{2max} for 30 min every day leads to a significant and prolonged dysfunction of the mitochondrial energy status of peripheral blood leukocytes, which is accompanied by an increased propensity for apoptosis and raised pro-inflammatory mediators (Tuan *et al.*, 2008). These results support the immunosuppressive effects of excessive exercise.

2.4.2 Effects of exercise on neutrophils

Exercise effects on immunity are highly dependent on exercise intensity, duration and frequency (Nieman and Pederson, 1999). Neutrophils appear to be most affected by prolonged periods of intense exercise training. Lower resting and post-exercise neutrophils activity has been reported in athletes from a variety of sports compared with non-athletes or within the same athletes across different phases of the training cycle. For instance, elite swimmers undertaking intensive training had a significantly lower neutrophils oxidative activity at rest than age- and gender-matched sedentary individuals, and that function was further suppressed during the period of strenuous training prior to national-level competition (Mackinnon, 2000). Moreover, the incidence of symptoms of upper respiratory tract infection increases during periods of endurance training. Nevertheless, in one study on swimmers, declining neutrophils activation was found to be not correlated with the appearance of URTI over 12 weeks of intense training (Mackinnon, 2000).

The lowest values were observed during the phase of highest training intensity and activity was partially recovered during a subsequent rest phase. In another study, neutrophils phagocytic activity (latex bead ingestion) and activation (ROS production) were shown to be significantly lower at rest and 24 h after a

standard maximal exercise test during intense compared with moderate training in distance runners, and compared with matched non-athletes (Mackinnon, 2000). The data suggest that down regulation of neutrophil function may occur in response to prolonged periods of intense exercise training. The biological significance of such changes is not currently known; although it has been speculated that down regulation of neutrophils function reflects an adaptive response to chronic inflammation resulting from tissue microtrauma elicited by daily intense exercise.

In a separate study, sedentary young males performed two months of moderate exercise (pedaling on the ergometer at a moderate intensity for 30 min each day) followed by two months of detraining (Syu *et al.*, 2012). It was found that at the end of the two months exercise period, researchers' noted increased chemotaxis, phagocytosis and citrate synthase activity of the neutrophils. In addition, the effects remained after detraining except for phagocytosis. Moreover, the fact that moderate exercise improves neutrophils functions may partially explain why physically active subjects have a low risk of infection.

In an animal study, researchers have evaluated the effects of moderate exercise on the neutrophils function in juvenile rats (Braz *et al.*, 2015). It was found that moderate exercise did not impair the viability and mitochondrial transmembrane potential of neutrophils, whereas there was greater reactive oxygen species production and phagocytic capacity. These results suggested that moderate exercise in juvenile rats improves neutrophils function, similar to adults.

2.5 Bee propolis, exercise, and immune function

To date, despites numerous beneficial effects of propolis found in animal and *in vitro* studies, there are very limited studies have been carried out to determine the effects of bee propolis supplementation on immune function among athletes.

In 2009, one study had investigated whether CAPE has protective effect against hyperthermal stress in athletes (Chen *et al.*, 2009). In this study, researchers isolated peripheral blood mononuclear cells (MNC) from competitive cyclists and assessed their response to hyperthermia with or without CAPE pre-treatment. They found that pre-treatment of cyclists' MNC with CAPE (0, 1, 2, 4 μg/mL) reversed or reduced hyperthermia-induced survival inhibition, necrosis, superoxide production, glutathione depletion and intracellular superoxide burst in a dose-dependent manner. These results suggest that CAPE may enhance the hyperthermal tolerance in immune mononuclear cells of competitive cyclists.

With lack of evidence to support the beneficial effects of bee propolis on immune function among athletes, hence, the present study is warranted.

CHAPTER 3

METHODOLOGY

3.1 Sampling and sample size calculation

The convenience sampling was used in this study. Participants were recruited among USM students. Sample size was calculated by using PS Software. Based on a study which was carried out by Muhammad and Gleeson (2013), the power of the study was set at 80% with 95% confident interval. The standard deviation (σ) observed was 0.2 (10^9/L) of monocyte counts, and difference in population mean (δ) was set at 0.2 (10^9/L) of monocyte counts. The calculated sample size was 10 participants. Considering 10% participants drop-out rate, 11 participants were recruited for this study. This study was conducted at the Sports Science Unit laboratory, USM. Method of inviting participants was through a poster advertisement (Appendix II).

3.2 Participants

Participants recruited in the present study were healthy males, aged between 18 and 25 years, non-smokers and running regularly at least 3 times per week with at least 30 min per session. In addition, the exclusion criteria includes having cold or respiratory tract infection at least two weeks prior to the study, having rhinitis, allergy to bee products, asthma or on medication. Throughout the study period, participants were required to abstain from taking any supplements that are known to affect immune function, e.g. probiotics, vitamin C and vitamin D. The

noncompliance rate to the supplementation's regimen was set at 20%; means that if participants' compliance is less than 80%, they will be withdrawed from the study.

3.3 Bee propolis supplementation

In the present study, participants consumed bee propolis tablets (500 mg of bee propolis/tablet) for 4 weeks, 2 tablets per day in the morning, as prescribed on the bottle. The bee propolis supplement was manufactured by Unison Nutraceutical Sdn. Bhd. and is commercially available in the pharmacy throughout Malaysia. Documents related to safety of the product were attached (Appendix III).

3.4 Study protocol

This study was a pre- and post- supplementation study design. During recruitment process, participants were given the participants' information sheet (Appendix VI) and were asked to fill in the consent form (Appendix VI) if they agree to participate. Then, participants performed 3 preliminary tests which include a submaximal test, a maximum oxygen uptake (VO_{2max}) test and a familiarisation test.

After that, participants performed the first exercise trial (90 min running at 60% VO_{2max}) after an overnight fast. This was followed by 4 weeks consumption of bee propolis where they had to consume 2 tablets per day. After the 4 weeks of consumption period, participants performed the second exercise trial which was carried out as similar as to the first exercise trial; 90 min running at 60% of VO_{2max} with an overnight fast again.

3.4.1 Sub-maximal test

This test was done to determine the relationship between running speed and oxygen uptake (VO_2). This is a 4-stage speed incremental test. It was done by setting the speed of the motorised treadmill at 6, 7, 8, and 9 kilometers per hour ($km.h^{-1}$). Participants had to run at each speed for 4 min after which the speed will be increased by 1 km at the end of each 4 min increment. During this test, oxygen uptake was recorded and a graph was plotted to observe the relationship of running speed and oxygen uptake.

3.4.2 Maximum oxygen uptake (VO_{2max}) test

This test required the participants to run to volitional exhaustion during a continuous incremental run on a motorised treadmill. Initially they performed warm-up for 5 min at a low speed (6 – 7 $km.h^{-1}$). After the warm-up, the participants were fitted with the head gear, mouth piece, nose-clip and heart rate sensor. An appropriate speed (8 – 12 $km.h^{-1}$) was selected and the test was begun with a grade of 0% for 3 minutes. Thereafter, the grade was increased 2.5° at every 2 min and the participants were encouraged to run until exhaustion. Oxygen uptake and heart rate responses were measured at the end of each 2 min stage. The VO_{2max} value was accepted to have been reached when there is a:

i) plateau in oxygen uptake despite increasing workload,

ii) failure of heart rate to increase with increases in exercise intensity, and

iii) respiratory exchange ratio of >1.15 (American College of Sports Medicine, 2000).

From the data obtained, running speeds during warm-up (50% VO_{2max}) and during running performance (60% VO_{2max}) was established from a regression equation with speed and oxygen uptake.

3.4.3 Familiarisation trial

This trial was carried out to make them familiar with the study protocol before they perform the actual experimental trials. It involved warm up by running at 50% VO_{2max} for 5 min followed by running at 60% VO_{2max} for 90 min. During the running, no blood samples were collected. However, oxygen uptake (VO_2) was recorded to ensure the speed given is at 60% of their respective VO_{2max}.

3.4.4 Experimental trial

Participants performed two exercise trials whereby the second exercise trial was performed after four weeks of bee propolis supplementation. The procedure of these two exercise trials was similar. On the experimental trial day, participants were requested to report at the laboratory at 7:30 am in the morning, after an overnight fast from 12 pm. Firstly, participants were cannulated for blood sample's collection. Then, the first blood sample (5 ml) was collected in an K_3EDTA tube. After that, participants performed warm up by running at 50% VO_{2max} for 5 min. Following that, participants ran at 60% VO_{2max} for 90 min. During the running trial, blood samples were collected after warm up, at min 30, 60, and 90. Heart rate, RPE, and oxygen uptake were measured before warm up, after warm up, and at min 20, 40, 60, 80, and 90 of the running trial. Furthermore, cool water as much as 3 mL per kg of participant's body weight was given to the participants at min 20, 40, 60, and 80 during the running trial. The final blood sample was collected 1 h post-exercise.

Blood samples were analyzed for neutrophils count. Figure 1 summarises the study protocol and Figure 2 summarises the experimental trial protocol.

3.5 Blood samples collection

Blood samples (5 ml) were taken from an antecubital vein by venipuncture with a 21 gauge needle into K_3EDTA collection tube using the vacutainer method. To make the vein become prominent, a tourniquet was used on the upper arm and was immediately released as soon as blood started to flow into the collection tube. Blood samples collected were used for measurement of neutrophils count.

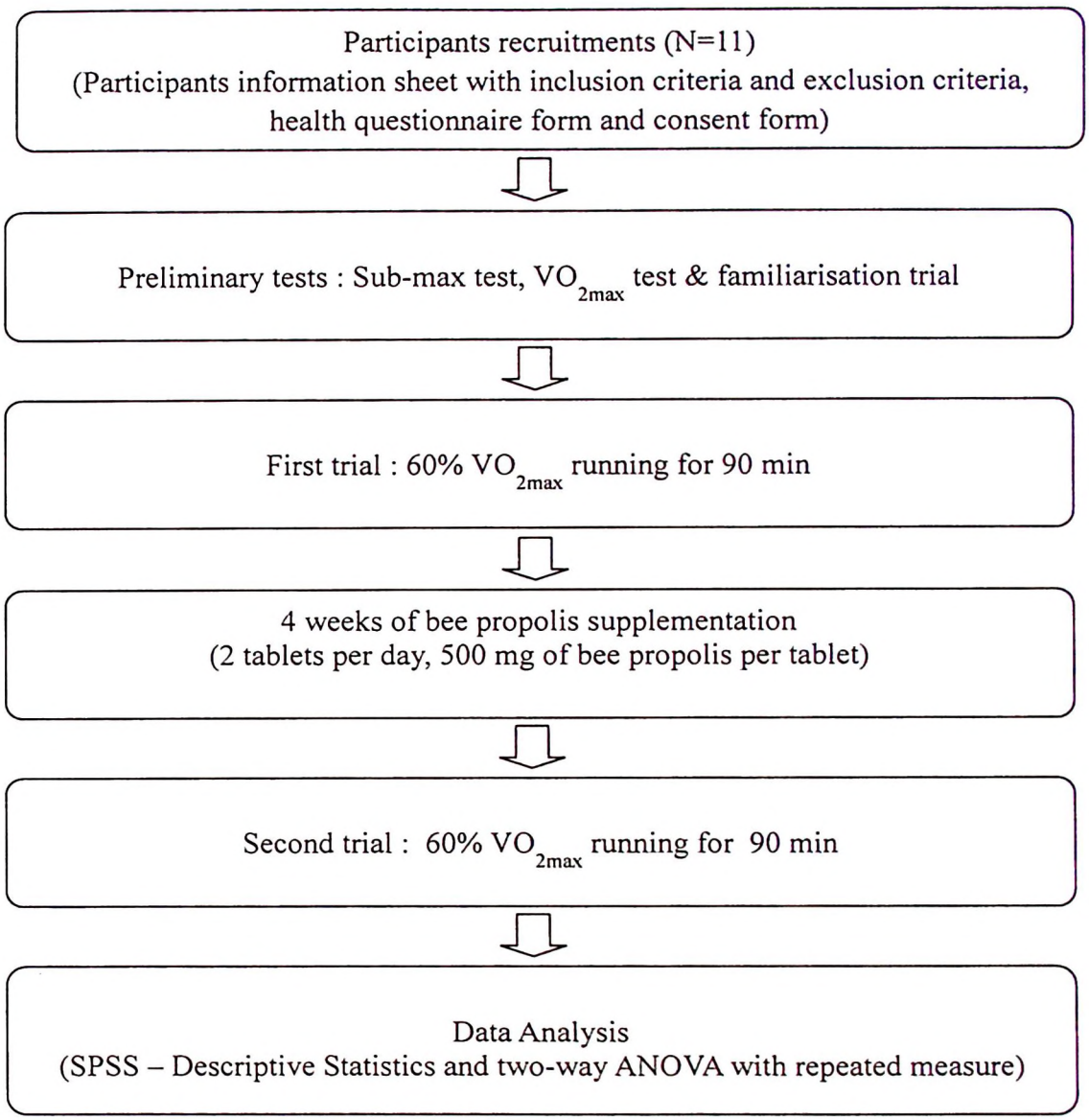


Figure 3.1: Flow chart of the study protocol

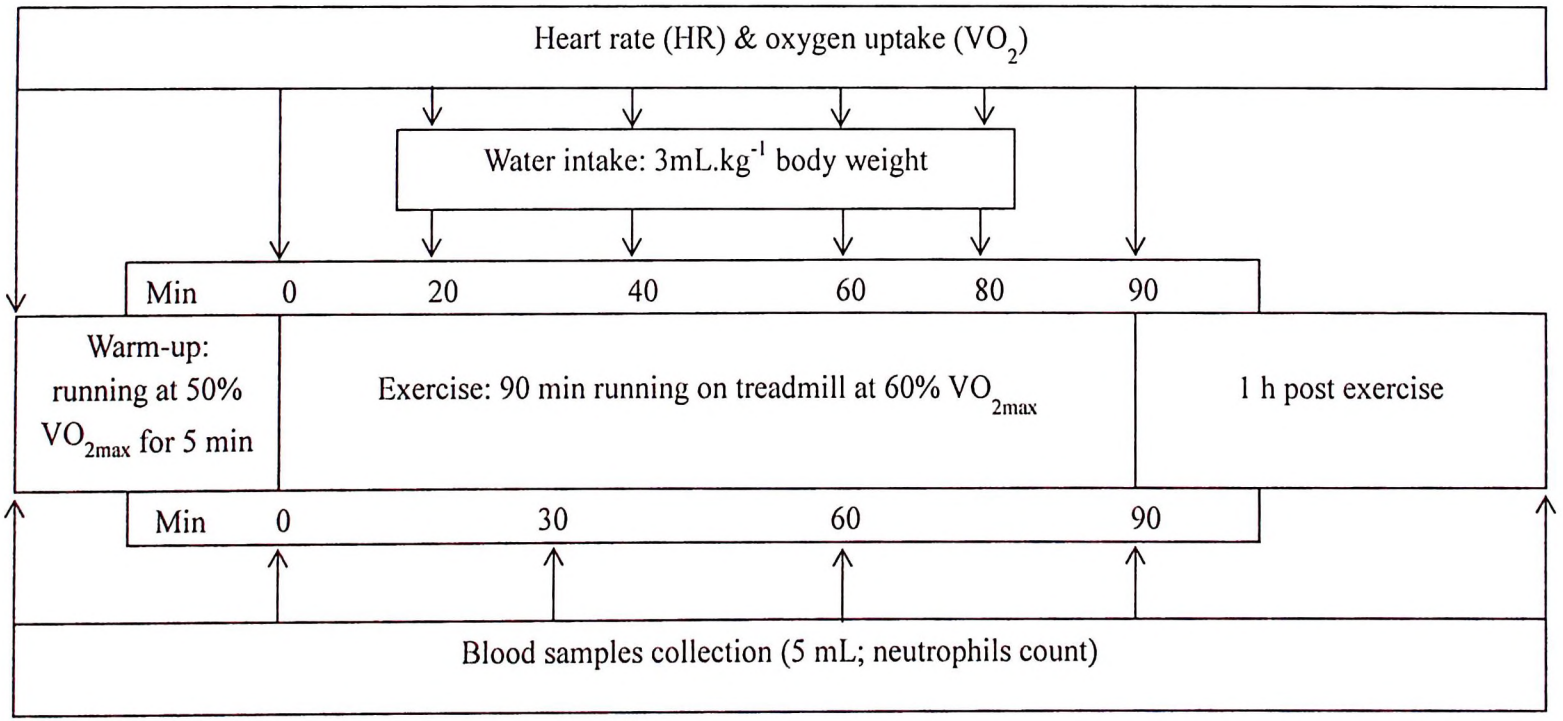


Figure 3.2: Flow chart of the experimental trial protocol

3.6 Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 22. All data were examined for normality through the Kolmogrov-Smirnov test. Descriptive statistics was performed on all dependent variables. Two-way ANOVA with repeated measures was performed to measure significant differences between trials and within trials. The accepted level of significance was set at $p < 0.05$. Results were reported as means ± standard deviation (SD).

CHAPTER 4

RESULTS

4.1 Anthropometry and physiological characteristics of the participants

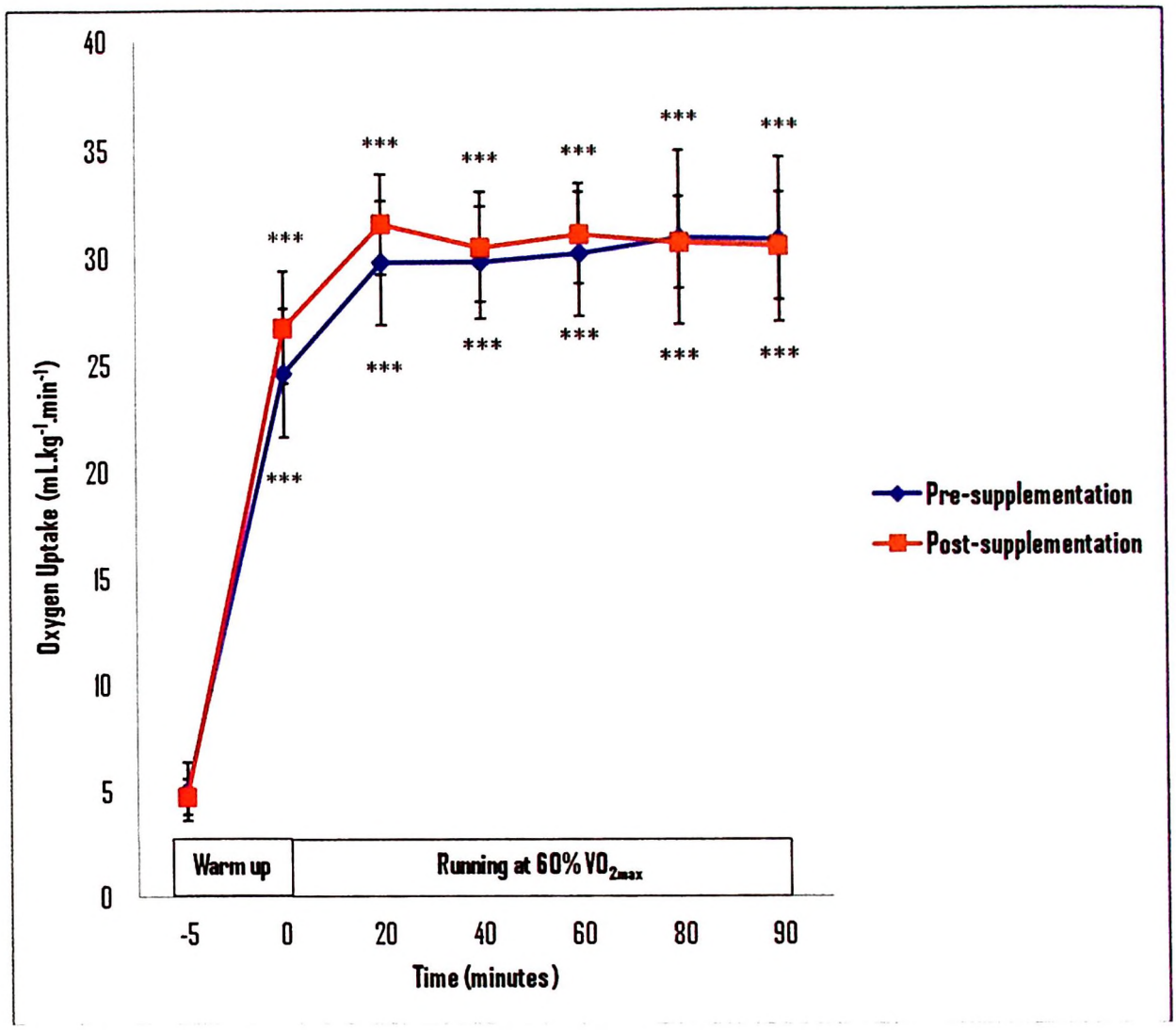
Table 4.1 showed the descriptive statistics which summarised the data of the anthropometry and physiological characteristics of the participants and were expressed in means ± standard deviation (SD).

Table 4.1 Anthropometry and physiological characteristics of the participants.

Parameters	Means ± SD
Age (years)	21.0 ± 1.5
Body weight (kg)	63.7 ± 7.2
Body height (cm)	169.3 ± 9.1
Body mass index (BMI) ($kg.m^{-2}$)	22.3 ± 2.5
Maximum oxygen uptake ($mL.kg^{-1}.min^{-1}$)	48.3 ± 4.4

4.2 Oxygen Uptake

The two-way ANOVA with repeated measures showed that there was a significant main effect of time on oxygen uptake during pre- and post-supplementation trials ($F = 470.7$; $df = 6$; $p < 0.001$) whereby it increased from baseline to the end of warm-up but then relatively stable throughout exercise (Figure 4.1). However, there was no significant interaction between time and trial on oxygen uptake ($F = 2.10$; $df = 3.74$; $p = 0.104$). It was also showed that there was no significant difference ($p > 0.05$) on oxygen uptake at any time point between trials. At the end of trial, oxygen uptake of the participants for pre- and post-supplementation trials was 31 ± 3.8 $mL.kg^{-1}min^{-1}$ (~60% of VO_{2max}) and 30 ± 2.5 $mL.kg^{-1}min^{-1}$ (~60% of VO_{2max}) respectively.



*** significantly different from respective resting values ($p < 0.001$).

Figure 4.1 Oxygen Uptake ($mL.kg^{-1}.min^{-1}$) in both pre- and post- supplementation trials.

4.3 Heart Rate

Based on the Figure 4.2, there was a significant main effect of time on heart rate during the trials ($F = 275.1$; $df = 2.6$; $p < 0.001$) whereby heart rate was significantly increased over time in both trials as analysed by the two-way ANOVA with repeated measures. Comparing the heart rate with respective resting values, it showed that there were significant differences ($p < 0.001$) in both trials. While comparing the time between trial on heart rate ($F = 1.91$; $df = 2.754$; $p = 0.155$), it showed that there was no interaction. In addition, there was also no significant difference ($p > 0.05$) on heart rate at each time points between trials. The heart rate of the participants for pre- and post- supplementation trials was 153 ± 12.3 and 150 ± 12.7 beats.min^{-1} respectively at the end of the trial.