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TUALANG HONEY'S SPERMATOPROTECTIVE EFFECTS IN A MODEL OF HIGH CHOLESTEROL DIET-INDUCED ANIMALS

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ABSTRACT

This investigation intended to ascertain the Tualang Honey's (TH) spermatoprotective effects on the reproductive system of male rats on a high-cholesterol diet (HCD). The animal study was conducted at the Department of Basic Medical Science, Kulliyyah of Medicine, IIUM. Twenty-four Sprague Dawley rats were fed with a 12% HCD for 16 weeks. Then, they were divided into four subgroups (group H1, H2, H3, and H4) and continued on the 12% HCD. This was conducted in addition to the administration of distilled water, 1.2, 2.4, and 3.0 g of honey per kg body weight daily, respectively for four weeks. By end of the fourth week, all rats were killed, and blood samples were brought for biochemical analysis as the testis and epididymis were gathered for histology and sperm analysis. All TH supplemented groups showed enormous improvements in the sperm parameters (sperm concentration, motility, progressive motility, normal morphology, and viability) in comparison to H1 ($P < .001$). The higher the TH dosage, the greater the improvements in the normal sperm morphology. In conclusion, the TH supplementation improved the sperm analysis results in the animal model, hence exerting the spermatoprotective effects.

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Introduction

Concerning the male reproductive system, cholesterol is thought to be essential not only for its function as a typical precursor for steroid hormone synthesis but also for regulating sperm function. Regulation of cholesterol homeostasis regulation is vital for post-testicular sperm maturation [1].

Nevertheless, an excessive intake of cholesterol-induced hypercholesterolemia will disturb cholesterol homeostasis in the body, which may cause adverse effects, including cardiovascular complications, obesity, metabolic disorders, and infertility [2, 3]. Hypercholesterolaemia is also linked to an upsurge in reactive oxygen species (ROS) which leads to the destruction of lipid peroxidation and deoxyribonucleic acid (DNA). This can disrupt spermatozoa and result in male infertility. Hypercholesterolaemia has been shown to adversely affect the post-testicular maturations and normal male reproductive functions and, therefore, predisposes to infertility. Nevertheless, the exact mechanisms are still poorly understood.

Tualang honey is wild multi-flowered honey generated by rock bees (Apis dorsata) that construct honeycomb high on the Tualang tree (Kompassia excelsa) branches. It owns a high reputation in Malaysia due to its medicinal benefits. Tualang honey is collected only by authorized bee-hunters under the supervision of the Malaysian Federal Agricultural Marketing Authorities (FAMA). The honey consists of about 200 component-like sugars (fructose, glucose, maltose, and sucrose) and a few other substances like organic acids, vitamins, minerals, proteins, flavonoids, phenolic acids, enzymes, and other phytochemicals. The unique properties of Tualang honey that make it distinct from other types of honey are that it contains more flavonoids and phenolic acids which exert anti-oxidative and anti-inflammatory effects [4].

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Tualang honey with its anti-oxidative and anti-inflammatory properties may improve male infertility caused by hypercholesterolemia. Nevertheless, there is still a scarcity of studies on the protective effects of Tualang honey on the sperm profile of a high-cholesterol diet. Not only that but there are also limited proven supplements available for male infertility problems. Therefore, this study was conducted to examine the effects of Tualang honey on the male reproductive system in hypercholesterolemia.

Materials and Methods

Animal

In this research, 30 male Sprague-Dawley rats, weighing 200-250g, were gained from A-Sapphire Enterprise, Seri Kembangan, Selangor. They have housed at 60 ± 5% relative humidity and 20 ± 2ºC, with a 12-hour light/dark cycle. Before the experiment, all rats were freely given water and access to commercial rat pellets for two weeks as an adaptation step for them to get used to the unfamiliar setting. The International University Islamic Malaysia Institutional Animal Care and Use Committee (IACUC-IIUM), IIUM, Kuantan campus (No. of IACUC Approval: IIUM / IACUC Approval / 2019 / (9) approved the experiment's animal handling procedures, treatments, and experimental protocols, and it adhered to the guidelines of the Malaysian Code of Practice for the Care and Use of Animal for Scientific Purposes.

Preparation of 12% Cholesterol Diet

As demonstrated in Equation 1, pure analytical cholesterol powder supplied by Nacalai-Tesque, Kyoto, Japan, was combined with commercial rat pellets in the form of powder. On average, the daily intake of cholesterol by human adults is about 1–2 percent [5]. The rat dose equivalent was calculated using the Km factor for a rat. The Km factor of 6 according to Reagan-Shaw was chosen [6]. The process involved grinding 1 kilogram of commercial rat pellets into fine powder form and mixing it with 120 grams of analytical pure cholesterol powder and 2 grams of cholic to yield a preparation containing 12 % cholesterol. All preparations were conducted every day to evade oxidative changes in cholesterol.

Cholesterol dosage for rats:
=Percentage of cholesterol daily intake in human x Km (Rat) =2% x 6 (1)
=12% cholesterol daily for rat

Tualang Honey

Tualang honey (AgroMas, Malaysia) was supplied by Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia.

Tualang Honey Dosage Preparation and Administration

Tualang honey was administered once daily by oral gavage. The honey doses (Equation 2) were adjusted daily based on the daily body weight of the rat. The honey doses (1.2, 2.4, and 3.0 g/kg/day) were administered by an equivalent dose calculation for rats based on the Km factor and human consumption in the area [6]. On average, the daily intake of honey by a 60 kg healthy adult is about 1 teaspoon daily which is equivalent to 12 g of honey daily. The consumption per kilogram was, therefore, 0.2 g (0.2 g/kg). The rat dose equivalent was calculated using the Km factor as shown below:

Honey doses for rat
= 0.2 g/kg/daily x Km (rat)
= 0.2 g/kg x 6 (2)
= 1.2 g/kg/daily

Study Design

The 24 Sprague-Dawley rats were fed with a 12% HCD for 16 weeks. At the end of the 16th week, the rats were divided into four subgroups (H1, H2, H3, and H4) and continued with the 12% high cholesterol diet in addition to the administration of distilled water, 1.2, 2.4, and 3.0 g of honey per kg body weight daily respectively as a supplement for four weeks.

Body and Organ Weight

Measurements of body weight were performed and recorded daily. Necessary adjustments were made to the Tualang honey dose administration as per body weight. After sacrificed, the testis and epididymis were immediately and carefully removed, rinsed with ice-cold sterile physiologic 0.9% sodium chloride, gently blotted on clean filter paper, and then weighed.

Lipid Profile

The analysis of lipid profile included total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL). The analyses were performed using a biochemical analyzer (AU680 Beckman Coulter Chemistry Analyzer). The biochemical analyzer was calibrated before using Beckman Coulter reagents for every parameter.

Hormonal Assay

Follicle-stimulating hormone (FSH) and testosterone in serum were measured by enzyme immunoassay utilizing kits in the market (Elabscience FSH Kit for rats and Abcam Testosterone kit for rats).

Sperm Analysis Procedures

Sperm Collection

To run the analysis on sperms, the right epididymis was utilized as a standard procedure. The cauda epididymis was cut and put in a petri dish consisting of 4 ml of sperm-washing medium, pre-incubated to 37oC. The cauda epididymis was finely minced with a scissor to release the semen. Then, the sperm suspension was incubated for 30 minutes at 37oC to allow the sperms to swim out before proceeding with sperm analysis. The sperms from cauda epididymis were used because these sperms have attained full maturity, motility, and a high potential for fertilization.

Sperm Concentration

Sperm concentration was carried out using a hemocytometer. The counting was done in the ruled squares on the slide. A volume of 10 µl was pipetted from the epididymal suspension and mixed with 90 µl distilled water to create a ten-fold dilution. Then, 10 µl from the sperm water mixture was placed into the counting chamber on the hemocytometer and covered with a coverslip. The number of spermatozoa with head and tail in five squares (four corners and the centre) in the central grid of both sides were calculated and averaged. Below is the formula used to obtain the sperm concentration (sperm count) in every ml. To gain the number of cells per ml in a diluted sample, the formula is multiplied by 10,000 [7].

Concentration of sperm:
= Number of spermatozoa in five squares X 10 (dilution) X 5 squares X 10000 (3)

Sperm Motility

Sperm motility was assessed immediately after 30 minutes of incubation to prevent the damaging effects of pH or temperature changes on motility. The slides and coverslips were pre-warmed at 37oC before assessment. A drop of epididymal sperm suspension was put on a microscope slide and concealed using a coverslip. At least 200 sperms were observed at 400x magnification using a microscope. Assessment of the sperm near the edge of the coverslip was avoided. The percentages of progressive motility and motile sperm were recorded. Progressive motility was represented by a percentage of progressively motile sperm to the amount of sperm count. Sperm motility was represented by the amount of motile sperm to the amount of sperm calculated [8].

Sperm Viability

The Eosin-Nigrosin (eosin Y 1% and nigrosin 10%) staining technique was employed to analyze sperm viability and morphology [9]. In a clean Eppendorf tube, an aliquot of 50 µL of sperm suspension was thoroughly mixed with 50 µL of Eosin-Nigrosin stain. 15 µL of the stained sperm mixture was shifted onto a glass slide and five smears were prepared for every rat. The glass slides were dried at room temperature. The coverslips covering the glass slides were placed with one drop of mounting medium before being observed under x100 oil immersion with a bright field microscope. Dead sperms would have a pink head whereas viable sperms would have a whitish or colorless head. About 200 sperms were scrutinized for dead and live cells, and their proportions were documented [10].

Sperm Morphology

For sperm morphology analysis, the same sperm smear for the sperm viability analysis was used. The sperms were scrutinized at x100 oil immersion magnification under an imaging microscope to assess sperm head, neck, and tail morphology. Then, 200 sperms were examined to classify them into normal or abnormal types of sperms. The abnormalities were also categorized into head or tail abnormalities [10].

Histological Analysis

The specimens from the testis were stained with Haematoxylin-Eosin. The slides were viewed under a light microscope (Olympus BX51) and analyzed for microscopic features using Johnsen scoring **(Table 1)**. Photographs of the microscopic features were captured using Olympus DP20.

Table 1. Johnsen testicular scoring [11].

Score	Description
1	No cells in tubular section
2	No germ cells but sertoli cells present
3	Only spermatogonia present
4	Only a few spermatocytes (<5) present
5	Many spermatocytes present

6	Only a few spermatids present
7	Many spermatids present
8	Only a few spermatozoa present
9	Many spermatozoa present but germinal epithelium are disorganized with marked sloughing or lumen obliteration
10	Complete spermatogenesis with many spermatozoa. Germinal epitheliums are organized with regular thickness leaving an open lumen

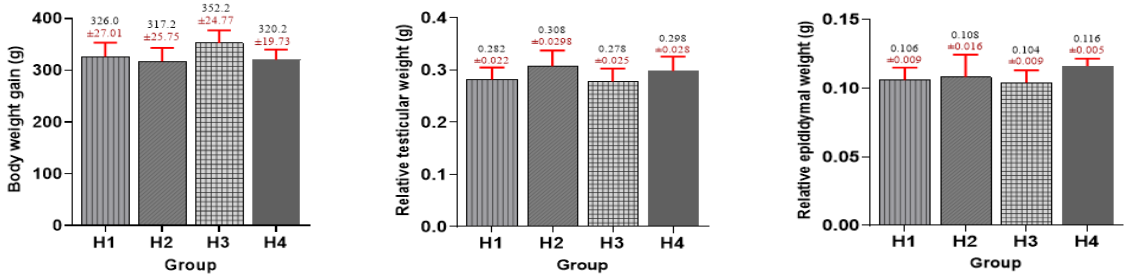
Statistical Analysis

The data from this study were analyzed using SPSS statistic software version 21.0. A statistically significant level was taken at a 95% confidence interval (P less than .05). The normally distributed numerical data were represented by the mean and standard deviation (SD). The mean comparisons were conducted by employing a one-way analysis of variance (ANOVA). The Post-hoc LSD and Tukey tests were used to determine the variances between every pair in the groups.

Results and Discussion

Results

Body and Reproductive Organs' Weight

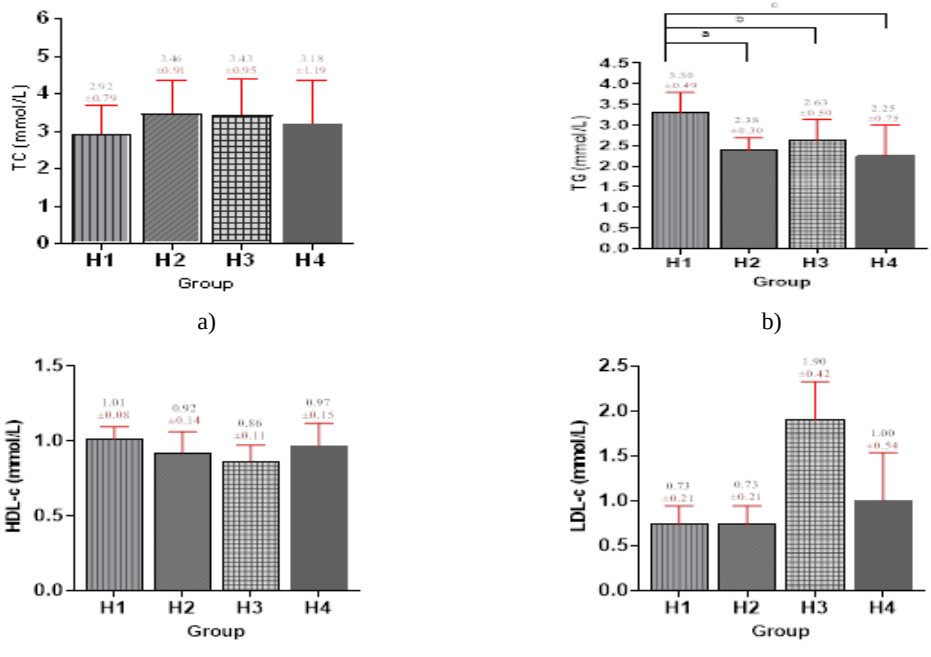


a) b) c)

Figure 1. Bodyweight gain, relative testicular weight, and relative epididymal weight in the high cholesterol diet groups.

Figure 1 shows the body weight gain, relative testicular weight, and relative epididymal weight in the high cholesterol diet groups. H1: Untreated high cholesterol diet; H2: high cholesterol diet with 1.2 g/kg/day of Tualang Honey; H3: high cholesterol diet with 2.4 g/kg/day of Tualang Honey; and H4: high cholesterol diet with 3.0 g/kg/day of Tualang Honey. No significant difference was found between the groups.

Lipid Profile



a) b)

c) d)

Figure 2. The lipid profile of the high cholesterol diet groups.

Figure 2 displays the lipid profile: total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) of the groups with high cholesterol diet. H1: Untreated high cholesterol diet; H2: high cholesterol diet with 1.2 g/kg/day of Tualang Honey; H3: high cholesterol diet with 2.4 g/kg/day of Tualang Honey; and H4: high cholesterol diet with 3.0 g/kg/day of Tualang Honey. There was a significant decrease in the serum TG levels of group H2, H3, and H4 (a, b, and c) compared to H1 (P=.026, P=.015, and P=.003, respectively).

Hormonal Assay
Follicular-Stimulating Hormone (FSH)

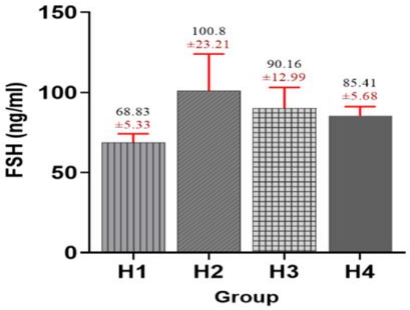


Figure 3. The level of follicular stimulating hormone (FSH) in the high cholesterol diet groups.

Figure 3 shows the level of follicular-stimulating hormone (FSH) in the high cholesterol diet groups. H1: Untreated high cholesterol diet; H2: high cholesterol diet with 1.2 g/kg/day of Tualang Honey; H3: high cholesterol diet with 2.4 g/kg/day of Tualang Honey; and H4: high cholesterol diet with 3.0 g/kg/day of Tualang Honey. No significant difference was found between the groups.

Testosterone
The level of testosterone could not be detected in all samples.

Sperm Parameters

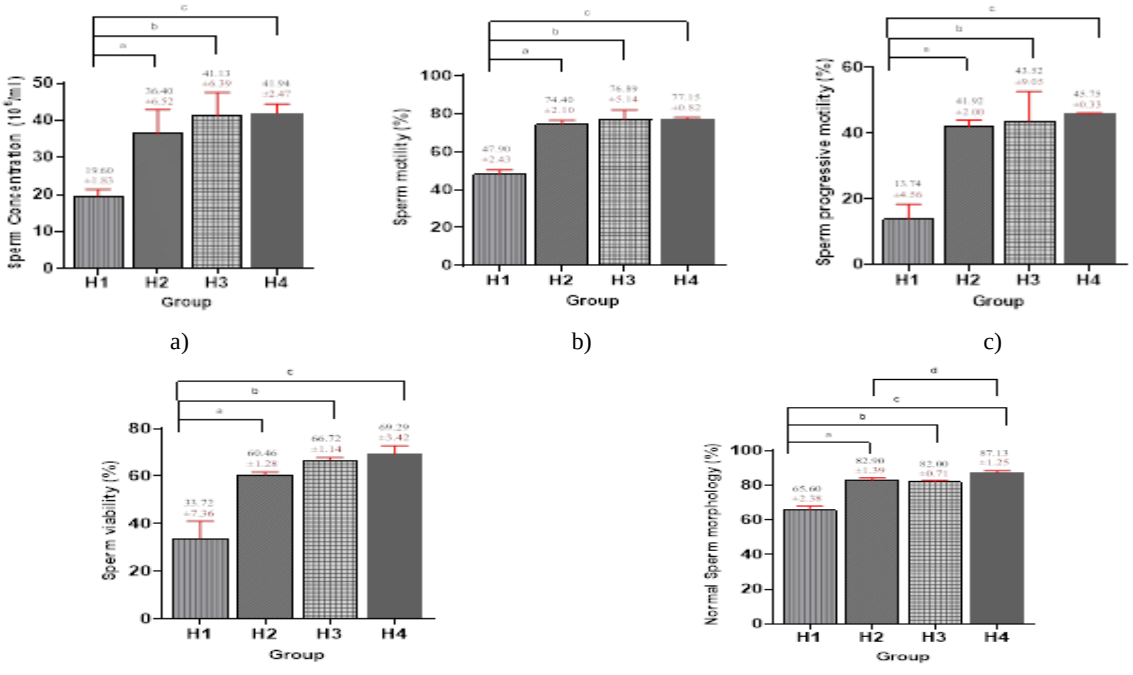


Figure 4. The sperm parameters in the high cholesterol diet groups

Figure 4 shows the sperm parameters in the high cholesterol diet groups. H1: Untreated high cholesterol diet; H2: high cholesterol diet with 1.2 g/kg/day of Tualang Honey; H3: high cholesterol diet with 2.4 g/kg/day of Tualang Honey; and H4: high cholesterol diet with 3.0 g/kg/day of Tualang Honey. A significant increase in all sperm concentration, sperm motility, sperm progressive motility, sperm viability, and normal sperm morphology was noted within all treated groups of high

cholesterol diet H2, H3, and H4 (a, b, and c) compared to H1 (all P<.001). Group H4 (d) significantly improved normal sperm morphology compared to group H2 with P=.005.

Histological Changes on Sperm Morphology

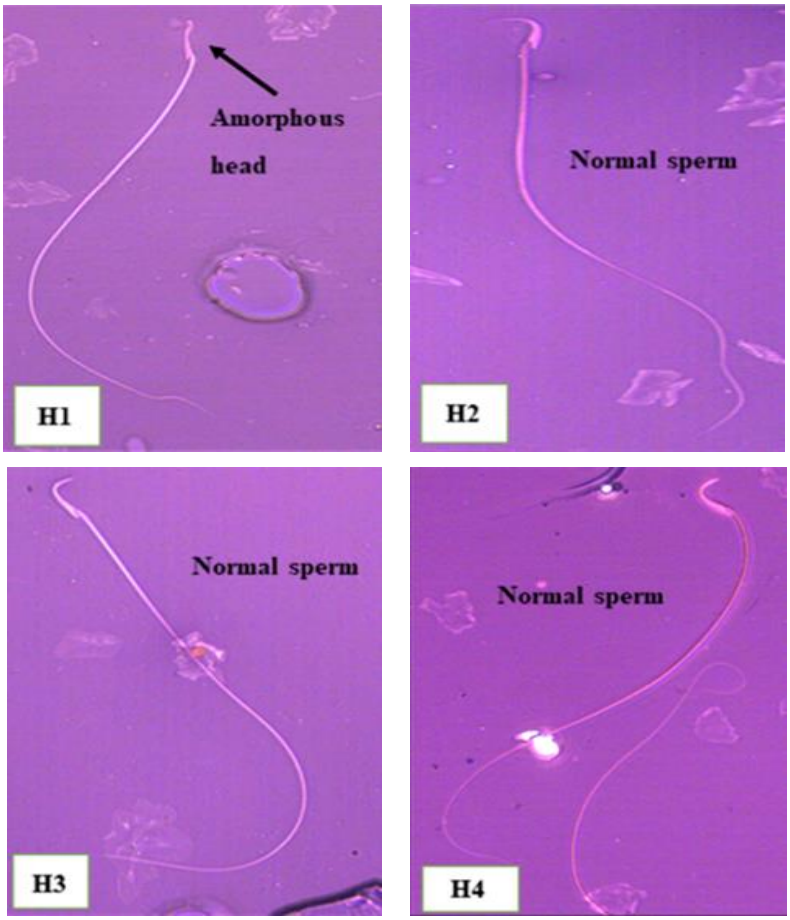
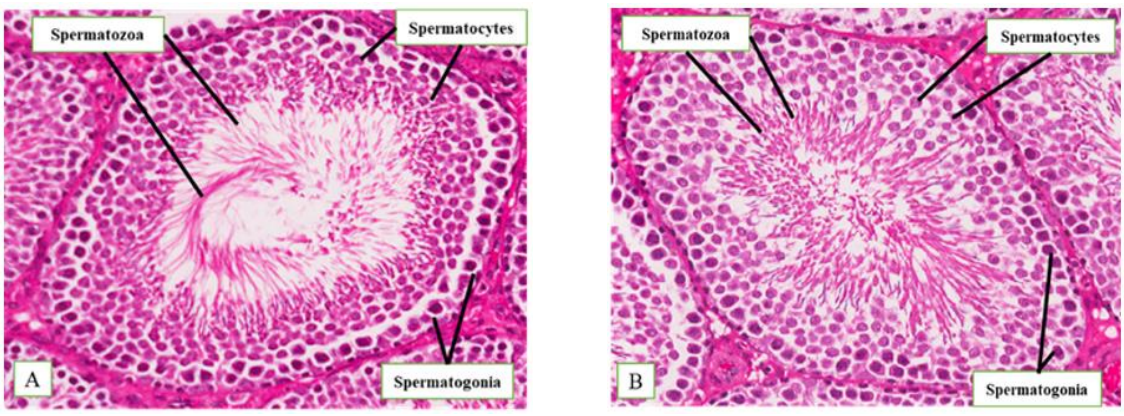


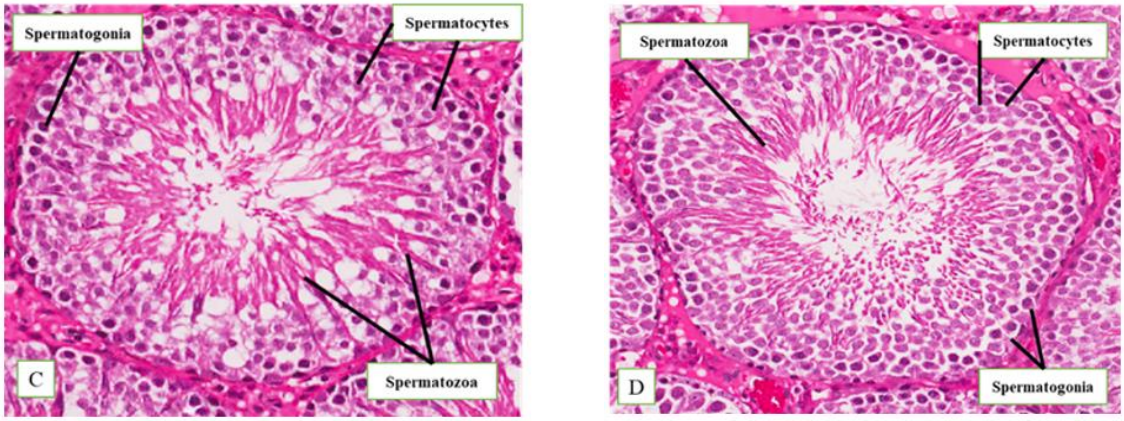
Figure 5. Eosin-Nigrosin stain for sperm morphology under 40x magnification; H1: Untreated high cholesterol diet; H2: High cholesterol diet + TH (1.2g/kg/day); H3: High cholesterol diet + TH (2.4g/kg/day); and H4: High cholesterol diet + TH (3.0g/kg/day). Sperms from H1 had abnormalities such as the amorphous head. Compared to H1, treated high cholesterol diet groups (H2, H3, and H4) had more percentage of normal sperm morphology.

The percentage of normal sperm morphology was increased in the H2, H3, and H4 groups as compared to H1.

Histological Analysis on Testis



a) b)



c) d)

Figure 6. The histology features of the Haematoxylin-Eosin stained seminiferous tubule of the left testis under 200x magnification from (a) H1= untreated high cholesterol diet; (b) H2 = high cholesterol diet with treatment dose 1 (1.2 g/kg/day); (c) H3= high cholesterol diet with treatment dose 2 (2.4 g/kg/day) and (d) H4= high cholesterol diet with treatment dose 3 (3.0 g/kg/day). The spermatocytes, spermatids, and spermatozoa were in the adluminal compartment with some spermatozoa present in the lumen. They had normal, full spermatogenesis.

Overall, there were no abnormalities observed in the histology of the left testis in all the groups. The Johnsen score was 10, which indicated full spermatogenesis with normal tubules.

Discussion

Honey has been found to have therapeutic potential in humans through multiple molecular processes related to oxidative stress, inflammation, and apoptosis, according to evidence-based studies [12]. this study, we have shown that the high cholesterol diet groups supplemented with Tualang honey (H2, H3, and H4) have significantly lower TG levels (P=.026, P=.015, and P=.003, respectively). All the three different concentrations of Tualang honey supplementation in high cholesterol diet improved all the sperm parameters (sperm concentrations, sperm motility, sperm progressive motility, sperm viability, and morphology) significantly with a P-value <.001. No prominent difference in the comparative weight of the testis between all groups was discovered in the present study. These findings are supported by several studies. In comparison to rats given sucrose solution, Nemoseck and colleagues discovered that rats given natural honey had lower TG levels and less weight gain [13]. Khalil *et al*. also reported that Tualang honey decreases total cholesterol and TG levels [14]. The natural ingredients of the Tualang honey contribute to its lipid-lowering properties. Polyphenols such as CA and p-coumaric acid (P-CA) possess a huge array of bioactive properties that include antioxidant, anti-inflammatory, and also lipid-lowering [15]. Some studies have reported that both CA and p-coumaric acid lower SREBP-1c and FAS mRNA expressions and impede their activities [16, 17]. SREBP-1c and FAS are both important regulators of free fatty acid (FFA) synthesis. The primary source of hypertriglyceridemia is dysregulation. In hepatocytes, FFA is the main compound in triglyceride synthesis. Lipolysis in adipose tissue rises in cases of hyperlipidemia and metabolic syndrome, resulting in increased FFA transport to the liver. This leads to the build-up of triglycerides [18, 19]. The high phenolic acid content in Tualang honey could reverse this action. Previous studies have shown that phenols could activate 50' adenosine monophosphate-activated protein kinase (AMPK), which will prevent SREBP-1c nuclear translocation. This will cause the reduction in SREBP-1c protein expression and hence inhibiting FAS synthase production. As a result, less free fatty acid will be synthesized, which leads to a decrease in triglycerides [16, 20].

A study done on Kelulut honey found that it increases the weight of testicles and epididymis in diabetic rats [21]. Nonetheless, in contrast with our study, no significant difference in the reproductive organs' weight was found. Mohamed and colleagues also reported a similar result: there was no significant difference in the weight of adult male Sprague Dawley rats' testis and epididymis which were treated with varied Tualang honey doses (0.2, 1.2, and 2.4 g/kg) for four weeks [22]. The inconsistent findings of the effect of honey on the weight of the reproductive organs in previous studies might be due to the species and sensitivity of animal used, the combination of diet given, different types and dosage of honey used, duration of treatment, and route of administration.

In addition, the results of the reproductive organs (testis and epididymis) weight, as well as the testis' histology (Johnsen scoring) might point out that honey was not harmful to these reproductive organs according to the doses used in this investigation for four weeks.

From our study, Tualang honey supplementation improved the sperm parameters of high cholesterol diet-fed rats. The findings in our study are in agreement with several studies. Tualang honey increased the sperm count, motile sperm percentage, and sperms with normal morphology percentage in the rats which were exposed to cigarette smoke [23]. Kelulut honey also revealed the possibility of escalating sperm motility and viability, including decreasing the abnormal sperm morphology percentage in the diabetic group which was treated with Kelulut honey [21]. Furthermore, similar effects also were seen in

humans. An experiment on oligospermia males revealed that sperm concentration, sperm motility, and normal sperm morphology were greatly enhanced by Tualang honey [24].

The exact mechanism of how Tualang honey improves the sperm parameters are still unclear. However, as mentioned previously, honey's antioxidant activity is the result of the interactions of several components. Kishore and colleagues reported that Tualang honey bees have a high level of radical scavenging activity, and possess the highest antioxidant activity of all the kinds of honey tested [25]. In a study done on male Sprague Dawley rats, Tualang Honey significantly increase anti-oxidant capacity, cause the supression of lipid peroxidation and stress-induced corticosterone [26]. Thus, it can be inferred that Tualang honey can reverse the oxidative stress as a result of high cholesterol diet and safeguard the germ cells. It has been documented that Tualang honey is high in flavonoids and phenolic acids. They may suppress oxidative stress because they can seek reactive oxygen species (ROS) molecules and prevent lipid peroxidation by constraining lipid alkoxyl radicals [4]. Flavonoids and phenolic acids also could interact with membrane phospholipids to form another protective surface on the membranes. As a result, this will sustain the membrane integrity by preventing the diffusion of ROS and lipid peroxidation by-products to the membranes. An interesting result was found in a study, where in the testicles, there was an increase in cholesterol content as well as a change in spermiogenesis. In the spermatozoa of HFD-fed mice, a disorderly manchette-perinuclear ring complex and an altered shape of the sperm head were found [27-29].

Modulation of seminiferous epithelium's cell cycle and enhancement of spermatogenesis by regulating spermatogenic cell proliferation are other possible explanations for the increase in sperm concentration [30]. Fructose and glucose are some of the components in Tualang honey. They are thought to provide more energy for sperm motility [31]. Besides that, Abdul Ghani and colleagues discovered that supplementing rats with Palestinian honey enhances epididymal sperm count, as well as testicular enzyme markers for spermatogenesis. They discovered that honey escalates the activities of sorbitol dehydrogenase, a testicular marker enzyme involved in spermatogenesis by 31% [32]. Besides that, honey also has been shown to improve the ability to get and sustain a penile erection [33]. Therefore, it can be concluded that all these mechanisms are responsible for reducing spermatozoa damage and dysfunction, thus improving the sperm parameters.

From the results, we found that the higher the dosage of Tualang honey given, the higher the improvement in normal sperm morphology. It is possible that the higher the dosage given, the higher the flavonoid and phenolic acid concentration, leading to a higher antioxidant activity. This will further protect the male reproductive system against oxidative stress and help to improve the sperm parameters in further exerting the spermatoprotective effects.

Conclusion

In summary, Tualang honey supplementation has proven to improve all the sperm parameters of the rat model of high cholesterol diet through its display of spermatoprotective effects. The higher the dosage of Tualang honey given, the better the improvement in normal sperm morphology. These are attributed to its high antioxidant properties. Therefore, Tualang honey exerts spermatoprotective effects in hypercholesterolemia animal models.

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Conflict of interest: None

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Ethics statement: The International University Islamic Malaysia Institutional Animal Care and Use Committee (IACUC-IIUM), IIUM, Kuantan campus (No. of IACUC Approval: IIUM / IACUC Approval / 2019 / (9) approved the experiment's animal handling procedures, treatments, and experimental protocols, and it adhered to the guidelines of the Malaysian Code of Practice for the Care and Use of Animal for Scientific Purposes.

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