RESEARCH REPORT (RIGS15- 078-0078)

Project ID/Title: MOLECULAR MECHANISM OF TUALANG HONEY ON 12% CHOLESTEROL DIET INDUCED NONALCOHOLIC STEATOHEPATITIS (NASH) ANIMAL MODEL

Project Sponsor: RESEARCH MANAGEMENT CENTRE, IIUM.

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Abstract: This study aimed to examine the effects of tualang honey against the high cholesterol diet induced biochemical and histological changes in the kidney, liver and pancreas. **Methods:** Female Sprague-Dawley rats were used where the control group (n = 5) was fed with commercial rat pellet; the high cholesterol diet (HCD) group (n= 5) was given 12% cholesterol diet; while the HCD with tualang honey (HCD+TH) group (n =5) was fed with 12% cholesterol diet with daily 1.4 g/kg/day of tualang honey. Biochemical analyses for lipid profile and renal function test were performed at completed 48 hours, 7 days, and 6 weeks. The rats were sacrificed at completed 6 weeks and the kidneys were harvested and subjected to histopathological examination. Blood biochemical analysis were also analysed at 1 week and 6 weeks for liver function test, fasting insulin, fasting glucose and HOMA-IR. The liver and pancreas tissues were harvested at the end of 6 weeks for histological examination. **Results:** The cholesterol diet induction resulted in dyslipidaemia and abnormal liver function. The HCD+TH group have shown an increase in total cholesterol, LDL-c, ALP levels and decreased TG, HDL-c and AST levels significantly at the end of 6 weeks compared to HCD group. Consumption of 12% cholesterol diet for six weeks resulted in an increment of the mean serum creatinine level of the HCD and HCD+TH groups to 1.5 times the control level at the completed 7 days. Also overall both the mean serum creatinine and blood urea levels were higher in HCD group than the control group. With tualang honey supplementation, the mean serum creatinine level showed significant reduction at 48 hours in the HCD+TH group as compared to the HCD group. There was also a reduction in the mean serum creatinine level at the completed 6 weeks. . Histopathologically the kidneys exhibited segmental mesangial hypercellularity and mesangial matrix expansion of almost all the glomeruli in both HCD and HCD+TH groups. The mean plasma glucose level was elevated at 1 week in the HCD group. Plasma insulin levels were higher in HCD+TH group at both 1 and 6 weeks. The HOMA-IR was also higher at 1 week in the HCD+TH group. The liver histology of both HCD and HCD+TH groups showed steatohepatitis with minimal hepatocyte degeneration while the pancreatic sections revealed no abnormalities. **Conclusion:** The 12% cholesterol diet of 6 weeks duration in this study did induce some features of NASH with dyslipidaemia with abnormal liver profile and also acute kidney injury. The tualang honey exerts some degree of renoprotective effect against high cholesterol diet induced kidney injury, but exhibited no effect on dyslipidaemia and histopathological changes in liver and pancreas.

Key words: High cholesterol diet, Tualang honey, kidney, liver and pancreas

Introduction

Kidney diseases include acute kidney injury (AKI), sub-acute kidney injury, and chronic kidney disease (CKD). For AKI more than 35 definitions have been used in the literature. This results in confusion and ill-defined association between acute renal dysfunction and morbidity and mortality (Mandelbaum et al., 2011). AKI is characterized by rapid and sometimes fatal loss of kidney function, leading to inability to maintain body fluids, electrolytes and acid-base homeostasis, and causing accumulation of end products of nitrogen metabolism (urea) and creatinine, or reduction in urine output, or both (Bellomo, Kellum, & Ronco, 2012). It is also defined as an abrupt reduction in the glomerular filtration rate (GFR) which results in accumulation of nitrogenous waste products, mainly creatinine and blood urea nitrogen (BUN) (Basile, Anderson, & Sutton, 2012).

However the preferred definition and staging system of AKI is the most recent proposed definition by the Kidney Disease: Improving Global Outcomes (KDIGO). According to KDIGO guidelines (2012), AKI defined as an increase in serum creatinine (SCr) by ≥0.3 mg/dl (≥26.5 μmol/l) within 48 hours; or increase in SCr to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or urine volume < 0.5 ml/kg/h for 6 hours (Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group, 2012). KDIGO guidelines proposed the term, acute kidney diseases and disorders (AKD), to include any decline in renal function occurring in less than three months. Disorders that evolve over more than 48 hours, but generally under than three months are referred to as sub-acute kidney injury. AKD includes both AKI and sub-acute kidney injury, and there is considerable overlap in an acute and sub-acute presentation (Pedram Fatehi, 2016).

The incidence of AKD is on the rise in both developed and developing countries (Lameire et al., 2013), and it has been proven that diet and lifestyle have an important role in its development. High cholesterol diet (HCD) has been documented to causes elevation of blood pressure and induce renal injury (Al-Rejaie, Abuohashish, Alkhamees, Aleisa, & Alroujayee, 2012). Researchers have proven that there exists a complex association between progressive renal damage and hypercholesterolemia (Ghada, 2014). These findings are of concerns as approximately 50% of the middle-aged adult population has been shown to have total cholesterol levels above the normal range (Chade et al., 2005). Most previous studies however focused on the impact of chronically high blood cholesterol levels on the renal tissue. One of them revealed that hypercholesterolemia resulted in the development of focal glomerulosclerosis and proteinuria that rapidly progressed to renal failure (Deepa & Varalakshmi, 2006).

In essence there is minimal information available in the literature with regard to early effects of hypercholesterolemia on the kidney (Abdel-Hafez, Othman, & Seleim, 2011). Also although there is a growing insight into the causes and mechanisms of the kidney diseases, preventive and therapeutic measures are still few (Lameire et al., 2013).

The persence of hepatic steatosis in the absence of significant use of alcohol or other liver disease is the characteristic feature of Nonalcoholic fatty liver disease (NAFLD). The progressive form of

NAFLD is known as Nonalcoholic steatohepatitis (NASH) (Vernonet al., 2011) which can be diagnosed by the presence of of steatosis, inflammation and hepatocellular ballooning on liver biopsies (Bruntet al., 2012). NAFLD is a leading cause to cirrhosis, liver failure, and hepatocellular carcinoma (Adams et al., 2005). Insulin resistance, oxidative stress and lipid peroxidation, proinflammatory cytokines, adipokines and mitochondrial dysfunction play an important role in the development and progression of NAFLD (Paschos & Paletas, 2009). In addition NAFLD is considered as the hepatic manifestation of the metabolic syndrome (TARGHER et al., 2007). NAFLD scoring system proposed by the Pathology Committee of the NASH Clinical Research Network comprised 14 histological features, 4 of which were evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4). Another nine features were recorded as present or absent (Kleiner et al., 2005). NAFLD is considered to be the most common cause of elevated liver enzymes (Vernon et al., 2011).

Honey has been used since ancient times to treat several diseases due to its medicinal importance (Tan et al., 2009). Tualang honey is a type of Malaysian polyfloral wild honey (Othman et al., 2015) rich in phenolic acids and flavonoid compounds which have strong free radical-scavenging activities (Khalil, et al., 2011). Tualang honey has a good anti-bacterial (Shehu et al., 2015) and anti-inflammatory activities (Al-Waili & Boni, 2003). It has a considerable effect on the healing process of different types of wounds (Khoo et al., 2010). It has been used in the treatment of diabetic foot (Imran et al., 2011). Tualang honey demonstrates a significant antioxidant activity (Shehu et al., 2015). It also has a significant anti-neoplastic activity (Yaacob et al, 2013) and a potential role in the improvement of learning and memory (Othman et al., 2015). It has cardioprotective (Khalil et al., 2015) and renoprotective (Mohamed et al., 2017) activities. In an experimental study, tualang honey produced a hepatoprotective effect (Erejuwa et al., 2011). As no drug therapy for NASH has been proved (Farrell & Larter, 2005), we aimed in this study to determine the possible hepatoprotective effects of tualang honey against high cholesterol diet induced NASH.

Objectives:

- To determine the acute and sub-acute effects of high cholesterol diet on the kidney in rat animal model
- To determine the protective effects of orally administered tualang honey on HCD induced AKD in animal rat model.
- To determine the effects of high cholesterol diet on the liver and pancreas biochemically and histologically.
- To examine the protective effects of tualang honey against the high cholesterol diet induced biochemical and histological changes.

Methodology:

Animals

Fifteen female Sprague-Dawley rats (age 6- 8 weeks) weighing 140 -170 grams were used in this study. The rats were purchased from A-Sapphire Enterprise, Seri Kembangan, Selangor. Two rats were housed in each cage under standard experimental conditions of 20 - 26°C at 50 - 70% humidity with 12 hours light/dark cycles. Throughout the experiment, the animals were given free access of water and food. The experimental protocols were approved by the Institutional Animal Care and Use Committee, International Islamic University Malaysia (IACUC-IIUM) No. of IACUC Approval : IIUM / IACUC Approval / 2016/ (12) (83).

High cholesterol diet

Twelve percent cholesterol diet was prepared by mixing 1kg of commercial rat pellet in powder form with 120 grams of analytical pure cholesterol powder (Nacalai-Tesque, Kyoto, Japan. Lot No. M4T5494. Code 08721-75). Three grams of cholic acid (Nacalai-Tesque, Kyoto, Japan. Lot No. M6H9123. Code 08805-56) were added to the preparation in order to produce stable hypercholesterolemia (Monte & Jimenez, 1993). In order to avoid oxidative modification of the cholesterol, the preparation of the high cholesterol diet was carried out on a weekly basis.

Tualang honey

Tualang honey (AgroMas, Malaysia) was supplied by Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia. The nutritional composition and specifications of tualang honey are as shown in table 1. The honey dose was calculated by conversion of human equivalent dose to rat dose using Km factor (Reagan-Shaw et al., 2008) according to as the following:

Human equivalent dose (HED) = Animal dose × Animal Km factor/ Human Km factor

(Reagan-Shaw et al., 2008)

Table 1: Nutritional composition and specifications of tualang honey

Parameter, Unit	Result	Standard (Food Reg 1985,Reg. 130)
Reducing Sugar (g/100g): Fructose Glucose	 38.0 36.9	>60.0
Sucrose (g/100g)	Not detected (<0.01)	<10.0
Ash (g/100g)	0.02	<1.0
Moisture (g/100g)	23.1	<20.0

Experimental design

Following 10 days of acclimatization, the animals were randomly divided into three groups. Group I served as a control group (n=5) and was fed with commercial rat pellet. Group II served as the high cholesterol diet (HCD) group (n=5) and was fed with 12% cholesterol diet. Group III (n=5, HCD+TH) was fed with 12% cholesterol diet along with oral daily dose of 1.4 g/kg/day of tualang honey by gavage. The experimental diets were administered for 6 weeks.

Biochemical study

Blood specimens collected at completed 48 hours, 7 days and 6 weeks were analysed for lipid profile and renal function test (Siemen Xpand Plus, USA).

Blood specimens were also collected, after overnight fasting, at 1 week and 6 weeks were analysed for liver function test (Siemen Xpand Plus, USA), fasting plasma glucose (MEDISAFE MINI Blood Glucose Reader, Japan) and fasting serum insulin (radioimmunoassay method). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the formula below:

HOMA1-IR = fasting serum insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5

(Matthews et al., 1985)

Histological study

At 6 weeks, all rats were euthanized and the kidney, liver and pancreas of each of the animals were harvested and fixed in 10% neutral buffered formalin for histological examination. The liver and pancreas tissues were processed using automated tissue processor (Leica TP 1020). The tissues were embedded into paraffin blocks (Leica EG1160). They were sectioned at 4 μm thickness and stained with hematoxylin and eosin (H&E) and Masson trichrome.

Statistical analysis

Statistical analysis was performed using ANOVA (SPSS version 20.0) to compare the biochemical blood results of the study groups. A value of $p<0.05$ was considered to be significant. The histological sections were analysed by two pathologists.

Results

Table 2. Lipid profile in the HCD and HCD+TH groups of rats

Lipid Profile parameter (mmol/L)	48-hours		7 Days		6 week	
	HCD	HCD+TH	HCD	HCD+TH	HCD	HCD+TH
Cholesterol	2.30±0.40	2.40±0.69	2.84±0.68	1.83±0.71*	7.38±2.93	8.76±4.07
HDL-c	1.88±0.21	2.03±0.32	2.45±0.65	2.11±0.95	0.23±0.10	0.18±0.08
Triglyceride	0.50±0.20	0.41±0.17	0.44±0.10	0.28±0.11*	0.53±0.15	0.53±0.11
vLDL-c	0.10±0.04	0.08±0.03	0.08±0.02	0.05±0.02	0.11±0.03	0.11±0.02

Values are given as means ± sd. Significant differences were analysed using Student's t-test, and indicated as *p<0.05 when comparing HCD with HCD+TH rats.

Table 3. Renal profile in the HCD and HCD+TH groups of rats

Renal Profile parameter	48-hours		7 Days		6 week	
	HCD	HCD+TH	HCD	HCD+TH	HCD	HCD+TH
Creatinine (µmol/L)	33.40±9.15	15.60±9.9*	25.20±12.80	25.40±10.03	40.60±4.28	34.80±3.83
Urea (mmol/L)	4.56±1.05	4.46±0.87	9.24±4.07	6.26±1.15	9.76±4.20	7.98±0.68
Sodium (mmol/L)	137.40±0.55	137.60±1.67	136.80±2.17	138.20±1.30	139.20±2.05	138.80±0.84
Potassium (mmol/L)	5.38±0.29	5.54±0.88	7.18±1.86	5.96±0.78	4.40±0.48	5.10±0.38
Chloride (mmol/L)	100.40±0.55	101.00±1.22	100.20±0.84	100.00±1.22	99.80±1.48	99.40±0.55
Uric acid (mmol/L)	0.11±0.05	0.26±0.26	0.16±0.04	0.19±0.07	0.13±0.03	0.13±0.02

Values are given as means ± sd. Significant differences were analysed using student t-test, and indicated as *p<0.05 when comparing HCD with HCD+TH groups of rats.

Table 4. Liver function test.

Liver profile parameters	1 week			6 weeks		
	Control	HCD	HCD+TH	Control	HCD	HCD+TH
Albumin (g/L)	13.4±0.55	12.80±1.79	12.80±1.92	37.8±1.30	39.40±2.07	40.00±3.39
Total bilirubin (umol/L)	3.40±0.55	3.40±0.89	3.20±1.10	2.00±0.00	2.00±0.00	2.00±0.00
Alkaline phosphatase (U/L)	231.40±42.88	343.40±77.80*	337.40±22.84*	144.40±32.85	200.60±46.67*	209.00±37.88*
GGT (U/L)	3.40±2.51	3.00±1.41	3.80±2.56	3.20±0.45	3.60±0.89	3.40±0.55
AST (U/L)	279.20±31.14	374.40±99.28	329.20±74.11	169.60±19.48	264.40±48.27*	200.80±40.21*
ALT (U/L)	53.60±3.65	48.00±23.84	62.60±19.42	47.80±3.96	54.80±7.76	52.80±9.68

Values are given as means ± sd. Significant differences were analysed using ANOVA test, and indicated as *p<0.05 when comparing control with HCD and HCD+TH groups.

Table 5. Fasting glucose, Insulin, and HOMA-IR

Parameters	1 week			6 weeks		
	Control	HCD	HCD+TH	Control	HCD	HCD+TH
Plasma glucose (mmol/L)	6.02±0.69*	7.82±1.80*	6.86±0.86	7.34±1.04	6.90±0.95	6.92±0.97
Serum insulin (uU/ml)	0.34±0.17*	0.46±0.15	0.68±0.16*	0.52±0.23*	0.56±0.17	0.78±0.13*
HOMA-IR	0.09±0.06*	0.16±0.06	0.20±0.05*	0.17±0.08	0.17±0.07	0.24±0.05

Values are given as means ± sd. Significant differences were analysed using ANOVA test, and indicated as *p<0.05 when comparing control with HCD and HCD+TH groups.

Renal histology

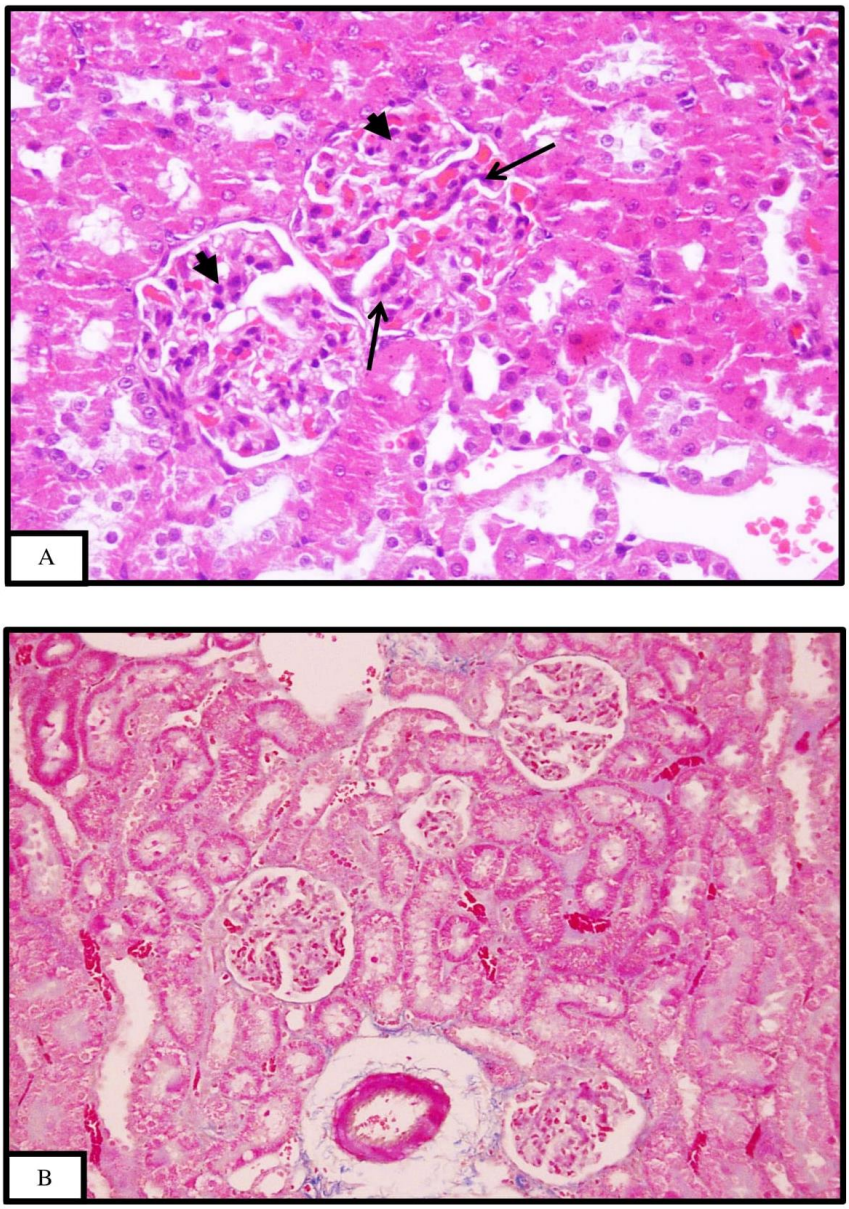

Figure 1: Pictomicrograph of a kidney section of the high cholesterol diet (HCD) group. Representative (A) H&E-stained section (x40 objective) showing segmental mesangial hypercellularity (arrow) with mesangial matrix expansion of the glomeruli (arrow head) and (B) Masson- trichrome-stained section (x40 objective) exhibiting of no area of increased amount of periglomerular or peritubular fibrous tissue formation.

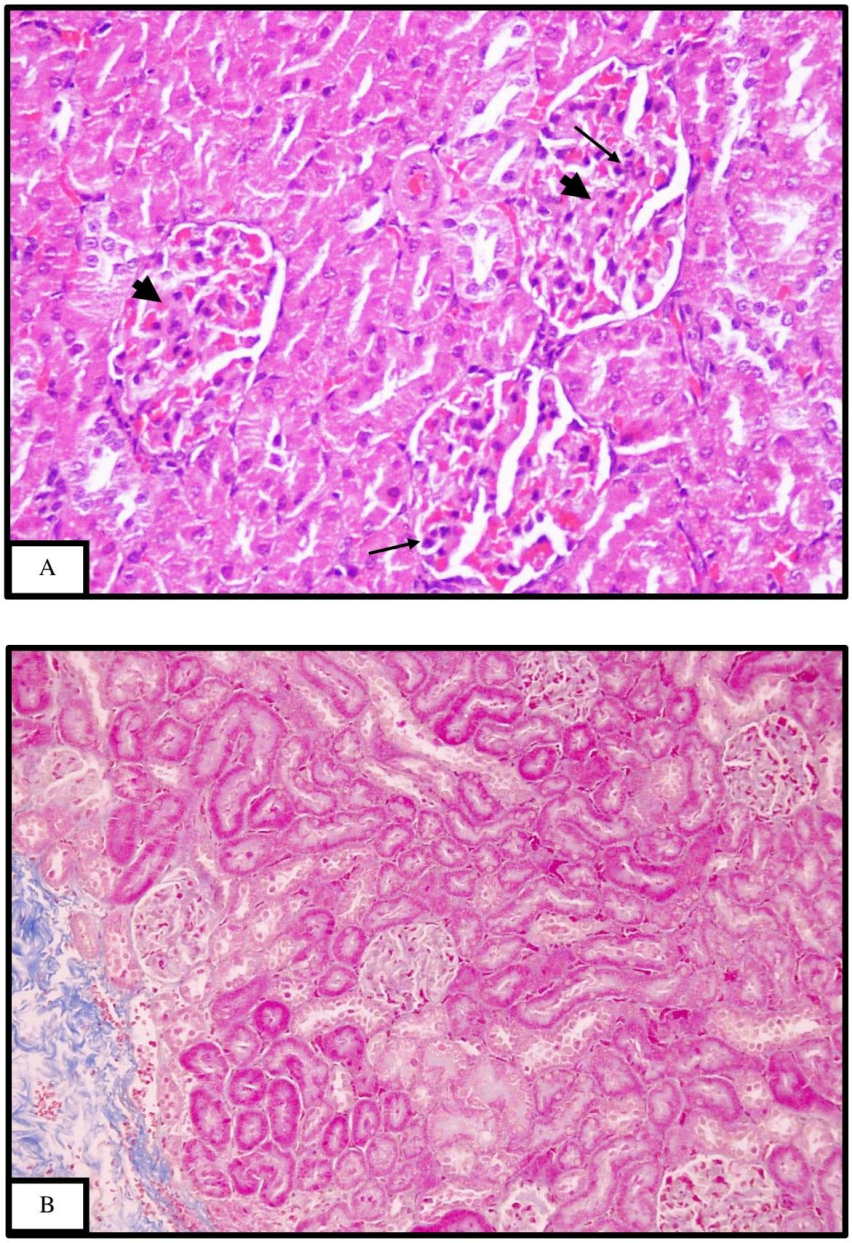

Figure 2: Pictomicrograph of a kidney section of the high cholesterol diet with tualang honey (HCD+TH) group. Representative (A) H&E-stained section (x40 objective) showing segmental mesangial hypercellularity (arrow) with mesangial matrix expansion of the glomeruli (arrow head) and (B) Masson trichrome-stained section (x20 objective) exhibiting of no area of increased amount of periglomerular or peritubular fibrous tissue formation.

Liver histology

The control group showed normal liver histology. The sections of the livers from the HCD group and HCD+TH group showed areas of microvesicular steatosis with mild lobular and portal inflammation (Figure 1). Hepatocyte degeneration was minimal. Sections of the liver stained with Masson Trichrome stain from all the groups revealed no areas of fibrosis (Figure 1).

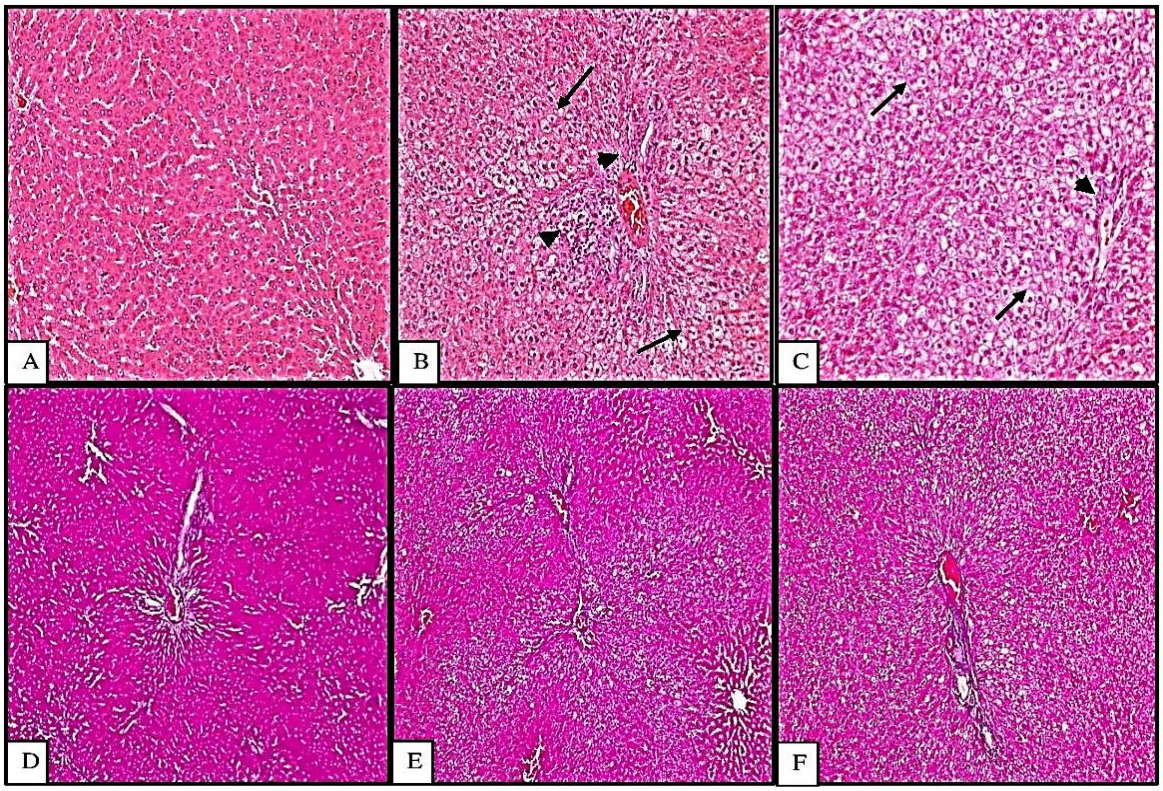

Figure 1: (A): Section of the liver from control group (Haematoxylin and Eosin stain, x20 objective). The section showed normal liver histology. (B): Section of the liver from high cholesterol diet group (Haematoxylin and Eosin stain, x20 objective). The section showed microvesicular steatosis (arrow) with lobular and portal inflammation (arrow head). (C): Section of the liver from high cholesterol diet with Tualang honey group (Haematoxylin and Eosin stain, x20 objective). The section showed microvesicular steatosis (arrow) with mild lobular inflammation (arrow head). (D): Section of the liver from control group (Masson Trichrome stain x10 objective). The section showed no area of fibrosis. (E): Section of the liver from high cholesterol diet group (Masson Trichrome stain x10 objective). The section showed no area of fibrosis. (F): Section of liver from high cholesterol diet with Tualang honey group (Masson Trichrome stain x10 objective). The section showed no area of increased of fibrous tissue formation.

Pancreatic histology

Sections of the pancreatic tissues from the control group, HCD group, and HCD+TH group all revealed normal pancreatic glands. Sections of the pancreatic tissue stained with Masson Trichrome stain from the three groups showed no areas of increased amount of fibrous tissue formation (Figure 2).

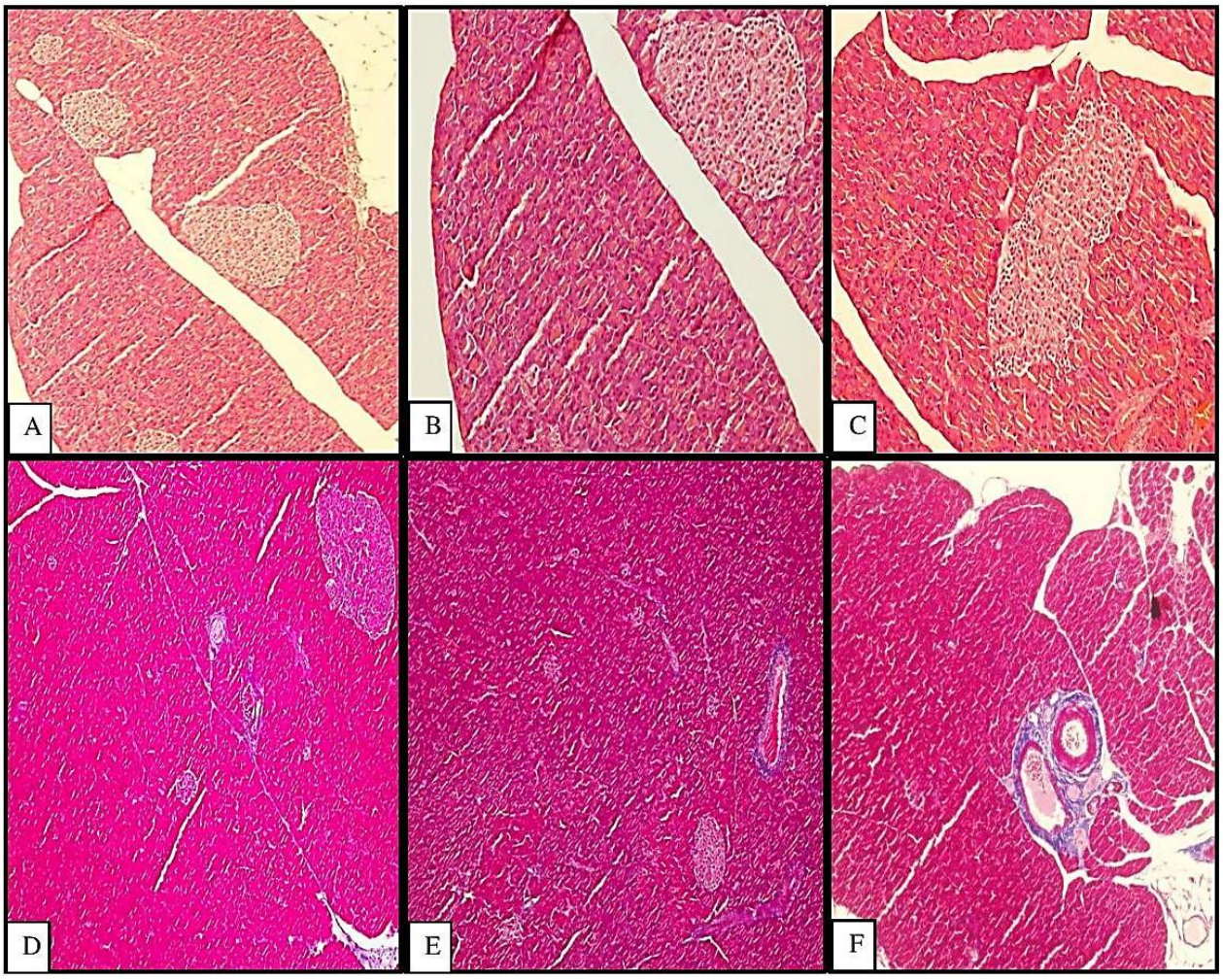

Figure 2: (A): Section of the pancreatic gland from control group (Haematoxylin and Eosin stain, x20 objective). The section showed normal pancreatic gland. (B): Section of the pancreatic gland from high cholesterol diet group (Haematoxylin and Eosin stain, x20 objective). The section showed normal pancreatic gland. (C): Section of the pancreatic gland from high cholesterol diet with Tualang honey group (Haematoxylin and Eosin stain, x20 objective). The section showed normal pancreatic gland. (D): Section of the pancreatic gland from control group (Masson Trichrome stain x10 objective). The section showed no area of increased amount of fibrous tissue formation. (E): Section of the pancreatic gland from high cholesterol diet group (Masson Trichrome stain x10 objective). The section showed no area of increased amount of fibrous tissue formation. (F): Section of pancreatic gland from high cholesterol diet with Tualang honey group (Masson Trichrome stain x10 objective). The section showed no area of increased of fibrous tissue formation.

Conclusion

In the present study tualang honey supplementation has resulted in an improvement of the renal profile suggesting therefore its renoprotective effect. However the histopathological examination of the kidneys revealed similar changes to that of the rat models fed with high cholesterol diet and this may be related to the dose of honey used in the study. Additionally tualang honey showed improvement in triglyceride and TC levels indicating its lipid lowering activities at 1 week.

The 12% cholesterol diet of 6 weeks duration in the animal model did induce some features of NASH with dyslipidaemia and abnormal liver profile but no effects were documented on the plasma glucose, serum insulin and HOMA-IR index. The pancreas also revealed no abnormality histologically. Tualang honey supplementation for the 6 weeks duration along with the 12% cholesterol diet resulted in improvement of the liver enzymes but exhibited no effect on the dyslipidaemia and did not improve the histopathological changes of the liver. Further studies are needed to explain the higher serum insulin levels and HOMA-IR with the honey supplementation. Also a longer duration of the high cholesterol diet administration would perhaps results in more obvious histological changes compatible to NASH. Additionally the dosage and duration of honey supplementation are subjected to further studies.

Output:

Thesis

1) Zenab B. Hamad Mohamed (2017). Renoprotective role of tualang honey against high cholesterol diet induced acute kidney diseases in female rat. Thesis International Islamic University Malaysia.

Research book

1) Zenab Hamad Mohamed, Roslina Abdul Rahim, Naznin Muhammad, Nor Zamzila Abdullah (2017) Effect of Tualang honey in acute kidney injury animal model. Lap Lambert Academic Publishing, Saarbrucken, Germany. ISBN 9783330070318.

Journal

1) Zenab Hamad Mohamed, Hamad Mohamed, Norra Harun, Naznin Muhammad, Nor Zamzila Abdullah, Roslina Abdul Rahim (2017). Renoprotective effects of tualang honey in high cholesterol diet induced acute and subacute kidney injuries in an animal model. J App Pharm Sci, 2017; 7 (12): 97-101.
2)

Conference Proceeding

1) Zenab Hamad Mohamed, Hamad Abdulsalam Hamad Alfarisi, Nor Zamzila Abdullah, Naznin Muhammad, Roslina Abdul Rahim (2017). Renoprotective role of tualang honey against high cholesterol diet induced acute kidney diseases in an animal model. Proceedings of the Medical Research Symposium 2017 International Medical Journal Malaysia (IMJM), 16 (supp. 1). , 0 pp. 26.
2) Zenab Hamad Mohamed, Hamad Abdulsalam Hamad Alfarisi, Nor Zamzila Abdullah, Norra Harun, Naznin Muhammad, Roslina Abdul Rahim (2017). Early effects of high cholesterol diet on the kidney of an animal model. Proceedings of the Medical Research Symposium 2017 International Medical Journal Malaysia (IMJM), 16 (supp. 1). , 0 pp. 20.

Future Plan of the research

We would like to prolong the induction time of high cholesterol diet and increase the dosage of Tualang honey treatment and study the gene, protein expression in kidney, liver and pancreas.

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