ORIGINAL ARTICLE

Anti-inflammatory Effects of Trihoney in Hypercholesterolemic Atherosclerotic Rabbits: A Comparative Study With Atorvastatin

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ABSTRACT

Introduction: Hypercholesterolemia has been proven as a main pathogenic trigger for pathogenesis of atherosclerosis. Atherosclerosis characterised by chronic inflammatory process and increased expression of inflammatory markers. In this study; Trihoney (a combination of three types of natural honey namely: Trigona, mellifera, and Dorsata) was investigated for its anti-inflammatory effect in hypercholesterolemic atherosclerotic rabbits. **Methods:** Thirty male New Zealand white rabbits (NZW) were grouped into: normal diet (C), normal diet with 0.6g/kg/day of Trihoney (C+H), 1% cholesterol diet (HCD), 1% cholesterol diet with 0.6g/kg/day of Trihoney (HCD+H), and 1% cholesterol diet with 2mg/kg/day of atorvastatin (HCD+At.). After 12 weeks of starting the experiment, animals were sacrificed and serum analysed for homocysteine and pro-atherogenic inflammatory markers such as: interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α). Fasting serum glucose was analysed to assess glycaemic status. **Results:** Trihoney treated group showed significantly lower ($p<0.05$) serum IL-1β and IL-6 compared to the HCD group. Trihoney supplementation resulted in significant ($p<0.001$) reduction of serum TNF-α compared to HCD group. Experimental group HCD had serum homocysteine level comparable to that of the control groups without any significant difference despite little increase in the mean value. Trihoney treated group had serum homocysteine comparable to the controls. All experimental groups showed fasting serum glucose comparable to the control. **Conclusion:** This study showed that Trihoney has an anti-inflammatory function and may be used as an adjuvant to statins for management of atherosclerotic cardiovascular diseases even in diabetic subjects.

Keywords: Atherosclerosis, Atorvastatin, Inflammatory markers, Trihoney

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INTRODUCTION

Atherosclerosis is characterised by chronic inflammatory process and increased expression of inflammatory markers (1). Inflammatory markers implicated in the pathogenesis of atherosclerosis are the pro-atherogenic cytokines (IL-1β, IL-6, TNF-α) (2), in addition to adhesion molecules such as: ICAM-1 and VCAM-1 which together with those cytokines play an important role in atherosclerotic plaque formation, and found to be associated with hypercholesterolemia (3,4). Homocysteine has been recognised as an independent risk factor for atherosclerosis and was linked to the pathogenesis of atherosclerosis through induction of endothelial apoptosis and dysfunction (5). Despite the continuous extensive work investigating drugs that can counteract the inflammatory processes in atherosclerosis, most of those drugs were found to have unavoidable side effects such as myalgia, and elevated liver enzymes (1). Honey has been shown to exert modulating activities on serum inflammatory marker TNF-α and some other inflammatory cytokines (6). Moreover, honey showed lowering effect on serum homocysteine level (7). In this work, authors investigated Trihoney for its anti-inflammatory effects on the key pro-atherogenic inflammatory cytokines and homocysteine that are implicated in the development and progression of atherosclerosis. Trihoney is a combination of three types of honey namely: Trigona, mellifera, and Dorsata. This product was made in the Department of Nutrition Sciences laboratories, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia (IIUM). The combination ratio was 45, 15, and 10 of the mentioned honey respectively and it was determined by Design Expert Version 6.0 software and Response

Surface Methodology (RSM) looking for a combination formula having the maximum total phenolic content (TPC) (unpublished data). Moreover, Trihoney had high concentrations of phenolic compounds such as: Quercetin, Kaempferol, Rutin, Cathecin, Maleic acid, Caffeic acid, Cinnamic acid, Coumaric acid, Gallic acid, p-Hydroxybenzoic acid, Salicylic acid, Sinapic acid, Vanillic acid, in addition to high antioxidant properties were reported through ferric reducing ability of plasma analysis (FRAP) and DPPH free radical scavenging activity analysis (unpublished data). Additionally, this combination suggested to provide synergistic effects between honey types with regard to the anti-inflammatory and immunomodulatory functions.

MATERIALS AND METHODS

Trihoney and atorvastatin

Trihoney was administered to the respective animal group by oral route at dose of 0.6g/kg/day. Dose has been calculated based on human and rabbit Km factors (8). Atorvastatin 40 mg film-coated tablets (Prague-Czech) were crushed into fine powder, reconstituted in 1 mL of distilled water and given by oral gavage using clean syringe (9–11), at a dose of 2mg/kg body weight.

Preparation of 1% cholesterol diet

Preparation of 1% cholesterol diet was performed as follows (10): 40 g of pure cholesterol powder (Nacalai-Tesque, Kyoto, Japan) was emulsified in 80 mL (=80 g) of cholesterol free extra virgin coconut oil (product of Philippines). The cholesterol emulsion was evenly poured over 3880 g of standard rabbit pellets (Perternakan Hong Lee Sdn. Bhd, Malaysia). The prepared food (1% cholesterol, 2% coconut oil rabbit pellet) then packed in zipped bags and kept at temperature of 20-22°C for use.

Animal

Thirty NZW rabbits of male gender were purchased from certified experimental animal supplier (A Sapphire Enterprise, Seri Kembangan, Selangor, Malaysia), animal weight ranged from 2 to 2.5 kg, and animals age 20 weeks. Animals were randomly housed in stainless-steel cages designed for rabbits as a single rabbit per cage with free access to water and standard rabbits' pellet in addition to the standard animal care housing condition of 12 hours dark/light cycle, temperature 15-21°C, humidity 45-65%. Procedure of animal handling was conducted in accordance with the guidelines of Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes (AEPC) (12), and the protocol of this experiment was approved by the Institutional Animal Care and Use Committee of International Islamic University Malaysia (IACUC-IIUM) with ID approval (IIUM/IACUC- Approval /2017 (19)).

Experimental study

Thirty male NZW rabbits were grouped into the following 5 groups: normal diet (C), normal diet with Trihoney dose of 0.6g/kg/day (C+H), 1% cholesterol diet (HCD), 1% cholesterol diet with 0.6g/kg/day of Trihoney (HCD+H), and 1% cholesterol diet with 2mg/kg/day of atorvastatin (HCD+At.). Treatment continued for 12 weeks. Blood withdrawal has been done from the central ear artery (13) after completion of the experimental period.

Blood samples and serum preparation

Blood has been collected from the animals into plain tubes, allowed to clot at room temperature for 40 minutes (14), and then centrifuged (Centrifuge Universal 320R Hettich, Germany) at 4°C by speed of 3500 rpm, for 15 minutes (15,16). The supernatant sera were collected and decanted into 200µL Eppendorf tubes and immediately stored at -80°C (Haier Ult Freezer, China) until analysed (17,18).

Enzyme linked immunosorbent assay (ELISA)

Pro-atherogenic inflammatory cytokines: IL-1β and TNF-α were determined quantitively using Cusabio (Hubei Province-China) ELISA kits for rabbits. Serum IL-6 was determined quantitively using Elabscience (China) ELISA kit. These assays employed the sandwich-ELISA principle. Serum homocysteine concentration was determined quantitively using homocysteine ELISA kit (Cell Biolabs, USA) and this assay employed competitive inhibition enzyme immunoassay technique. All ELISA procedures were conducted according to the manufacturers' protocols.

Serum glucose analysis

Fasting blood glucose, and fasting insulin were measured before commencement of treatment as well as the end point of the experiment. Samples were immediately analysed by automated analysis machine (Au480 Au Analyser-Beckman Coulter, Inc.).

Statistical analysis

Statistical Package for Social Sciences (SPSS version 21 Chicago, Illinois, USA) software was used for data processing. Data were expressed as mean (M) and standard deviation (SD) and analysed by one-way analysis of variance (ANOVA). One-way ANOVA followed by Post Hoc test used for determination of significant differences between means of two or more independent groups. Statistically significance considered at $p< 0.05$.

RESULTS

Trihoney supresses pro-atherogenic inflammatory cytokines

Tremendous significant increase in serum IL-1β was seen in HCD group following 12 weeks treatment with 1% cholesterol diet (Table I). The HCD group showed significant ($p<0.001$) increase in serum IL-1β seen when compared to both C and C+H groups. On the other hand, Trihoney treated group HCD+H showed significantly ($p<0.05$) lower serum level of IL-1β when

Table I: Effects of 1% cholesterol diet and Trihoney on serum pro-atherogenic inflammatory cytokines

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
C	85.99±5.84	6.37±0.68	15.09±0.32
C+H	83.08±10.42	6.56±0.34	14.20±0.21
HCD	167.07±13.36$^{a,\ b***}$	7.88±1.78	16.64±2.09$^{a*,\ b**}$
HCD+H	130.30±28.05$^{a*,\ b**,\ c*}$	5.91±0.41^{c*}	12.74±1.37$^{a**,\ c***}$
HCD+At.	85.99±5.84^{c***}	6.95±2.00	13.90±0.18^{c**}

Values are mean ± standard deviation (SD) of the mean. The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD Post Hoc test. Mean difference is considered significant at (p<0.05). a= significant different from C group, b= significant different from C+H group, c= significant different from HCD group. [*= p<0.05, **=p<0.01, ***=p<0.001].

compared to the HCD group. Animal group HCD+At. which received atorvastatin exhibited serum IL-1β result very close to that of the C and C+H groups, in addition to a very significant (p<0.001) reduction in serum IL-1β compared to the HCD group. Serum IL-1β level in Trihoney treated group was comparable to that of the HCD+At. group. Very significant (p<0.001) positive correlation found between serum level of IL-β and aortic lesion area percentage. Likewise, serum IL-1β had significant (p<0.01 and p<0.05) positive correlation with serum total cholesterol (TC) and serum low-density lipoprotein cholesterol (LDL-c) levels respectively (Table II). Serum IL-6 level was higher in the HCD group compared to all other experimental groups. Trihoney treated group HCD+H showed significant (p<0.05) reduction in serum IL-6 level in comparison to the HCD group. The HCD+At. group had a lower serum IL-6 level in comparison to the HCD group even it was statistically not significant but it was very close to that of the control groups. The HCD group demonstrated a significant (p<0.05) elevation of serum TNF-α compared to the C group. Group supplemented with Trihoney exhibited serum TNF-α significantly (p<0.001) lower than that of the HCD group. Atherogenic group treated with atorvastatin showed significant (p<0.01) lower serum TNF-α level compared to HCD group.

Trihoney is a cardiovascular protective against hyperhomocysteinemia

The 1% cholesterol diet group had serum homocysteine level comparable to that of the control groups without any significant difference despite little increase in the mean value. Atherogenic group supplemented with Trihoney showed serum homocysteine level comparable to that of the control groups. Likewise, HCD+At. group exhibited serum homocysteine concentration comparable to the control groups but significantly (p<0.05) lower than that of the HCD group (Table III).

Table II: Correlation between serum interleukin-1β (IL-1β), and serum total cholesterol (TC), serum low-density lipoprotein cholesterol (LDL-c), and aortic lesion area percentage

	TC (mmol/L)	LDL-c (mmol/L)	Aortic lesion area percentage (%)
Serum IL-1β (pg/ml)	r=0.611**	r=0.502*	r=0.839***

Values are the Pearson correlation coefficient (r) between interleukin-1β (IL-1β), lipid profile, and aortic lesion area percentage (%) of the all experimental groups. [*= p<0.05, **=p<0.01, ***=p<0.001 Sig. (2-tailed)].

Table III: Effect of Trihoney and 1% cholesterol diet on serum homocysteine

Group	Serum homocysteine (µg/ml) week 12
C	4.65±2.29
C+H	4.13±1.14
HCD	5.29±1.62
HCD+H	4.26±2.28
HCD+At.	1.94±0.34^{c*}

Values are mean ± standard deviation (SD) of the mean. The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD Post Hoc test. Mean difference is considered significant at (p<0.05). a= significant different from C group, b= significant different from C+H group, c= significant different from HCD group. [*= p<0.05, **=p<0.01, ***=p<0.001].

Trihoney maintains normoglycemic status

Analysis of fasting serum glucose of all experimental groups showed no significant difference (p>0.05) after 12 weeks of treatment (Table IV).

Table IV: Effect of Trihoney on serum glucose

Group	Fasting serum glucose (mmol/L) week 12
C	5.45±0.82
C+H	5.20±0.98
HCD	5.05±0.94
HCD+H	5.52±1.01
HCD+At.	5.27±0.73

Values are mean ± standard deviation (SD) of the mean. The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD Post Hoc test. Mean difference is considered significant at (p<0.05).

DISCUSSION

Interleukin-1β and IL-6 are pro-atherogenic cytokines secreted by macrophages, smooth muscle cells (SMCs), and lymphocytes (2). The role of these cytokines in atherosclerosis is implicated in atherogenesis, endothelial adhesiveness and in induction of inflammatory markers (19). Among all cytokines , IL-1β has the most crucial role in inflammatory diseases (20). Interleukin-1β induces synthesis of IL-6 and TNF-α, and stimulates endothelial adherence of leukocytes through upregulation of intercellular adhesion molecule-1 (ICAM-1) (21). Interleukin-6 stimulates proliferation and differentiation of B and T lymphocytes, regulates the expression vascular endothelial growth factor (VEGF) (22), and contributes to atherosclerotic plaque development and plaque destabilisation (23). High circulating levels of TNF-α was found to have an association with the extent of atherosclerotic lesions in patients having acute coronary syndrome (ACS), and based on this role, TNF-α has been nominated as a pro-atherogenic cytokine (2,23).

In our study, feeding NZW rabbits for 12 weeks with 1% cholesterol diet resulted in significant elevation of serum IL-1β, IL-6 and TNF-α. These findings are supported by other hypercholesterolemic animal models that investigated the role of these pro-atherogenic inflammatory cytokines in the setting of hypercholesterolemia (3,24–27). In this experiment, we reported that Trihoney caused significant reduction in the serum level of IL-1β in hypercholesterolemic rabbits. This interesting finding may indicate the protective function of Trihoney against atherosclerosis progression. Moreover, this result indicated the anti-inflammatory property of Trihoney. Interleukin-1β is described as the master cytokine of inflammation in a broad spectrum of diseases including ACS (28), thus reducing its serum level in hypercholesterolemic model by using Trihoney may suggest this natural product as promising adjuvant therapeutic option to prevent or for minimise the inflammatory cascade in atherosclerosis.

Our results showed significant positive correlation between serum IL-1β level and the percentage of aortic atherosclerotic plaques, and this finding may indicate the pathogenic importance of IL-1β for the development of atherosclerotic plaques. Experimental studies showed practical evidence for the direct relationship between IL-1β and the atherosclerotic plaques progression. Inhibition of IL-1β was found to be associated with inhibition of atherosclerotic plaque formation, whereas increased IL-1β activity resulted in lesion progression (29). We reported positive correlation between serum IL-1β, serum TC and LDL-c. This correlation is supported by the natural history of pathogenesis of atherosclerosis following hypercholesterolemia (30). We demonstrated significant reduction in serum level of IL-6 when Trihoney was supplemented simultaneously with the atherogenic diet. This finding strongly supported the anti-inflammatory and the immune modulating role of Trihoney in the case of hypercholesterolemia and suggesting its protective function against the process of atherosclerosis.

Researchers work extensively to find medicines that are able to block inflammatory process mastered by these pro-atherogenic cytokines. Some of the medicinal drugs are approved and some are under clinical trials (28, 29). Despite the evidence of medical improvement in some of the examined inflammatory diseases, investigated drugs are associated with some serious side effects (29).

In this investigation, we showed significant reduction in serum TNF-α in Trihoney supplemented group. The potential of Trihoney towards reduction of TNF-α in this experiment was very comparable to or to some extent more marked than atorvastatin. The reported reduction of pro-atherogenic inflammatory cytokines in our study is in line with investigation conducted by Hussein et al. (2012) who reported reduction in IL-6 and TNF-α, both in serum and skin tissue following treatment with the Malaysian Gelam honey in rat model (31). Clinical trial study involving daily oral supplementation with natural honey for 10 weeks showed decrease in inflammatory cytokines IL-6 and TNF-α (32), which is in agreement with our findings. One more interesting finding about honey and cytokines in wounds is that, honey can induce and can inhibit cytokine production depending on wound condition (33). In vitro studies showed that honey had stimulatory effect on cytokine production (34, 35). In a recent randomised controlled trial study using Malaysian Tualang (Dorsata) honey supplemented to chronic smokers for 12 weeks in order to examine its effect on inflammatory cytokines, authors reported that honey causes opposite effects on high sensitivity C-reactive protein (hs-CRP) and TNF-α. Smokers who received honey had high TNF-α and low hs-CRP post-intervention compared to pre-intervention and authors attributed these conflicting effects to the nonconclusive effects of Tualang honey on those two inflammatory markers (36). The anti-inflammatory effect of Dorsata honey against hs-CRP most likely attributed to the phenolic compounds present in honey (36).

The possible anti-inflammatory mechanisms exerted by honey may operate through the nuclear factor kappa B (NF-kB) which has been implicated as a key inflammatory factor. Gelam honey has been reported to inhibit the nuclear translocation and activation of NF-kB and subsequently caused reduction in expression of proinflammatory mediators tumour necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2), thereby reducing the inflammation (37). Natural honey has been shown to reduce serum levels of prostaglandins (PGE2, PGF2α, and thromboxane B2), and this inhibitory effect found to increase with time, the site of action may involve COX-2, COX-1, or both (38). The anti-inflammatory effects of Trihoney can also be attributed to nitric oxide, vitamin E, prostaglandins, and phenolic compounds present in this mixture (39).

In our study, we used atorvastatin as a positive control because it is a well-known anti-atherosclerotic agent (40) with protective and therapeutic functions (41). The treatment group receiving atorvastatin had reduced serum levels of IL-1β, IL-6 and TNF-α. Atorvastatin treated group showed very close serum IL-1β to that of the control groups C and C+H. This finding indicated the anti-inflammatory effect of atorvastatin in hypercholesterolemia induced atherosclerosis. In line with our results, Loppnow et al. (2011) demonstrated an in vitro study of the inhibitory function of atorvastatin on the release of IL-6 in (SMC)/human mononuclear cell (MNC) coculture atherosclerotic model (42). This inhibitory effect of statins on IL-6 was also described by in vivo study looking for anti-inflammatory functions of statins (43). In agreement with our findings, in vitro study has demonstrated the anti-inflammatory ability of atorvastatin to downregulate IL-1β, IL-6, and TNF-α (42). Despite that, the effects of statins on these cytokines

are still controversial. Lyngdoh et al. (2011) conducted a cross-sectional study investigating the association between statins and the inflammatory cytokines, and they reported no association found between statins and the cytokines (IL-1β, IL-6, and TNF-α), which contradicts our findings (44). This contra version may be explained by Jameel et al. (2013) who mentioned the heterogenic effect among different statins towards the investigated cytokines, and concluded that not all statins are able to reduce inflammatory cytokines (44). Different mechanisms have been postulated to explain the observed anti-inflammatory function of statins. Recently, some authors suggested mechanisms such as the leukocyte adhesion cascade as central for the anti-inflammatory effect of statins through HMG-CoA reductase dependent and independent mechanisms in addition to other mechanisms away of statins' lipid lowering function (40, 43, 46, 47).

As far as hyperhomocysteinemia is concerned, high serum homocysteine level has been recognised as an independent risk factor for atherosclerosis (5). Hyperhomocysteinemia probably induces CVDs through some mechanisms including vascular endothelial damage, stimulation of SMCs proliferation, lipid peroxidation, and activation of thrombosis (48). In this work, feeding NZW rabbits with 1% cholesterol diet for 12 weeks failed to induce significant hyperhomocysteinemia in the HCD group. This finding adds to the debate on the association between homocysteine and hypercholesterolemia. Bolayirli et al. (2007) in hypercholesterolemic rabbits model showed a significant increase in serum homocysteine level following 8 weeks of supplement with high cholesterol diet (13). On the contrary, Momin et al. (2017) by cohort study conducted on 4660 subjects who were not on hypolipidemic treatment showed no significant association between serum homocysteine and TC or LDL-c which supports our study (48). In a clinical study involving 667 subjects who have familial combined hyperlipidaemia, the association between homocysteine and hyperlipidaemia was investigated, but no association between serum homocysteine level and serum lipids was found (49), which is in line with our experimental results.

In our experiment, Trihoney treated group showed serum homocysteine level comparable to the control groups, and lower than the high cholesterol diet group. Based on this finding, taking in consideration the result of C+H group which also received the same Trihoney dose, we may postulate that Trihoney has cardiovascular protective potential against hyperhomocysteinemia. In agreement with our work, honey was reported to reduce serum homocysteine level by 8% in normal subjects after 15 days of honey consumption (7). El-Saleh (2006) showed that natural honey had protective effects against hyperhomocysteinemia (50).

Interestingly, atorvastatin treated group in this study showed the lowest serum homocysteine level compared to all other treated and untreated experimental groups. No significant difference was reported between atorvastatin treated group and the control and HCD+H groups. On the other hand, this group had serum homocysteine level significantly ($p<0.05$) lower than the HCD group. This report is not in line with Bolayirli et al. (2007) who reported that atorvastatin unexpectedly increased serum homocysteine level in hypercholesterolemic rabbits (13). Our results showed that Trihoney may be comparable to atorvastatin in the setting of protection against hyperhomocysteinemia.

As far as serum glucose is concerned, treatment with Trihoney for 12 weeks had not induced disturbance of blood glucose, in other word Trihoney is not diabetogenic agent.

CONCLUSION

Trihoney exhibited anti-inflammatory effects by inducing significant reduction of serum pro-atherogenic inflammatory cytokines: IL-1β, IL-6 and TNF-α. Trihoney had neutral effect on serum homocysteine level and maintained a normoglycemic status. Trihoney has the potential cardioprotective and anti-atherosclerotic effects and may be suggested as an adjuvant remedy for management of atherosclerotic vascular diseases.

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