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Effects of neem (*Azadirachta indica*) leaf extracts on lipid and C-reactive protein concentrations in cholesterol-fed rats

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Abstract

Objectives: Aim of this study is to investigate the effects of Neem (*Azadirachta indica*) leaf extract on serum lipid parameters and C-reactive protein (CRP) concentrations. The association between CRP and serum lipid profiles in hypercholesterolemic rats is also investigated. Methods: Total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride (TG) and CRP were measured at week 0, 2, 4, 6 and 8 in 4 groups of animals; the control group (C), cholesterol control group (CC), cholesterol-fed groups treated with Neem leaf extract at doses of 50 and 300 mg/kg/day orally (N1 & N2), respectively. Results: Cholesterol feeding had resulted in a significant increase ($p<0.05$) in TC, LDL & TG in group CC starting from week 2 and a decrease in HDL levels ($p<0.05$). CRP concentrations also showed a significant increment in the cholesterol-treated rats (CC) compared to group C, N1 & N2 ($p<0.05$). Neem extract treated, TC, LDL and TG levels remained within the normal range similar to Group C and CRP concentrations were lowered but HDL levels were not. Conclusions: It is shown in this study that the concentrations of CRP had increased in CC rats but not in rats with normal cholesterol levels. Neem extract at 50 and 300 mg/kg doses had prevented the rise of TC, LDL and TG in cholesterol-fed rats. No significant changes in CRP concentrations were noted in the Neem-treated animals. Association between CRP and serum lipid concentrations is directly proportionate. They also suggest that Neem leaf extracts at 50 and 300 mg/kg concentrations are excellent lipid-lowering agent.

Keywords: Neem; C-reactive protein, Serum lipid; Hypercholesterolemia

1. Introduction

Recent studies showed that the risk of developing heart diseases and atherosclerosis were directly related to hypercholesterolemia [1,2]. Chronic elevation in blood cholesterol will lead to endothelial injury and eventually the formation of atherosclerotic plaques.

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Atherosclerotic plaques formation involves a complex series of cellular events similar to those that occur in chronic inflammation. Inflammation appears to be pivotal in all phases of atherosclerosis from the fatty streak lesion to acute coronary syndromes [3].

An important downstream marker of inflammation is C-reactive protein (CRP) [4]. Numerous studies have shown that CRP levels predict cardiovascular disease in apparently healthy individuals.

Azadirachta indica A. Juss., commonly known as Neem, is an indigenous species of the tropical countries, especially India and South East Asia. Neem leaf have been shown to be non-toxic and found to possess anti-inflammatory [5,6], hypoglycemic [7], antioxidant [8] and potent immunostimulant activity [9].

It is thought that due to its proven anti-inflammatory property, Neem might prevent inflammations from occurring in experimental hypercholesterolemia. Therefore, in the present study, we evaluated the effect of Neem leaf extract on cholesterol-fed rats.

2. Materials and methods

2.1 Plant material

The leaves of *Azadirachta indica* were procured from around the state of Selangor, Malaysia and were identified for their correct botanical identity and deposited at the Phytomedicinal Herbarium, Institute of Bioscience, Universiti Putra Malaysia, Selangor. (Voucher No.: SK 587/03 *Azadirachta indica*)

2.2 Preparation of extract

The leaves were oven-dried at 50°C for three days and ground into powder-form with a grinder. For the preparation of the ethanolic extracts, a total of 900g powder of the plant was extracted using 95% ethanol through condensation process using a Soxchlet Apparatus. The extract was then concentrated using a rotary evaporator and freeze dried to calculate a final concentration of 50 and 300 mg/kg body weight.

2.3 Experimental animals

Male Sprague-Dawley rats weighing approximately 200g procured from the animal house of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The animals (three per cage) were maintained under standard laboratory conditions with access to food and water *ad libitum*. All rats received approximately 20g of standard rat pellets per day, with or without cholesterol added. All experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee regulations.

2.4 Induction of hypercholesterolemia

The effects of Neem leaf extracts on normal and cholesterol-fed rats were studied for 8 weeks. All rats were subjected to a trial period prior to cholesterol feeding to estimate daily food requirement. They were divided into four groups with 6 rats per group; the control group (C), cholesterol-fed control group (CC), cholesterol-fed groups treated with Neem leaf extract at doses of 50 and 300 mg/kg/day orally (N1 & N2), respectively.

Group C continued to be fed a basal diet (cholesterol-free) for the next 8 weeks whereas group CC, N1 & N2 were given cholesterol-enriched diet (normal pellet + 3% cholesterol). Approximately 0.4 ml of olive oil was used to liquefy the ethanolic extracts of Neem leaf and fed to group N1 and N2 daily via oral gavage for 8 weeks. All the other rats also received the same amount of olive oil but without the extracts.

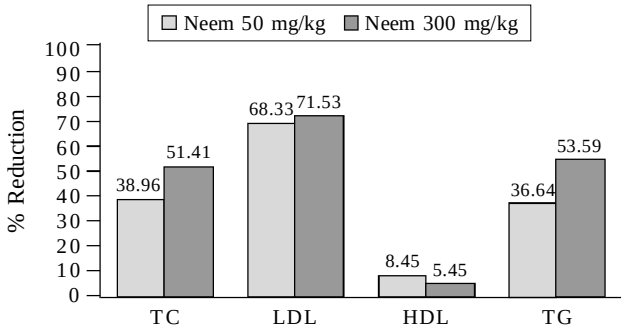

Fig. 1.
Percentage reduction of serum cholesterols and trygliceride by neem leaf extracts.

Values are percentage reduction when compared to cholesterol control group (CC) at week 8 (Refer to Table 1).

2.5 Analysis of serum lipids and C-reactive proteins

Approximately 2 ml of blood samples were taken from each rat at beginning of experiment and at week 2, 4, 6 and 8. Blood was taken via cardiac puncture using 23G needles and 3 ml syringes and collected into SSTII tubes (plain tubes). The sera were immediately separated by centrifugation at 3000 rpm for 10 min. They were then transferred into eppendorf tubes and stored under -80°C for further analysis.

Total cholesterol (TC), low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides (TG) were determined enzymatically using Hitachi 902 automated analyzer. Serum C-reactive protein (CRP) was determined by latex particle-enhanced immunoturbidimetric assay on a Hitachi 902 automated analyzer, by use of anti-CRP antibodies coupled to latex microparticles reacting with the antigen in the sample to form an antigen/antibody complex. Agglutination was then measured turbidimetrically.

2.6 Statistical analysis

Data are expressed in means (± S.D) where appropriate and analyzed statistically by using two-way ANOVA and those at $p<0.05$ are accepted as significant. A Tukey-Kramer post test is used to further evaluate the differences among the groups.

3. Results

Table 1 shows the serum cholesterols and triglyceride profiles. Cholesterol-feeding had resulted in significant increase in TC, LDL and TG. In CC, the rise in TC could be seen starting from week 2. By week 8, TC had increased 1.5-fold in the CC group, whereas TG and LDL had increased approximately 2-folds. HDL levels were significantly reduced in the CC group after 6 weeks of cholesterol-feeding. The serum cholesterol levels of rats given Neem leaf extracts were, however, significantly reduced as compared with those of the hypercholesterolemic control rats. At the end of week 8, the TC, LDL and TG levels in Neem-treated groups were comparable to those from the control group and from the week 0 values of the two groups (N1 & N2).

As shown in Fig 1, 50 mg and 300 mg/kg of Neem leaf extract had reduced 68.33 and 71.53% of LDL as compared to CC group, followed by TC (38.96% and 51.41%) and TG (36.64% and 53.59%), respectively. The percent reduction of HDL was very little (8.45 and 5.45% respectively) and the changes were not significantly different from control (Table 1).

As compared to normal rats, the CRP level was significantly elevated in CC group at week 6 and 8 of cholesterol-feeding (Fig. 2). The administration of Neem extracts, however,

prevented the CRP levels from rising in cholesterol-fed rats, at week 6 ($p<0.05$, Neem 50 mg/kg vs. CC group) and week 8 ($p<0.05$, Neem 50 & 300 mg/kg vs. CC group).

Table 1
Serum cholesterol and trygliceride values (mM/L) in control and neem-treated rats. Values are mean ± S.D. (*n*=6)

	Control (C)	Cholesterol Control (CC)	50 mg/kg Neem (N1)	300 mg/kg Neem (N2)
Week 0				
TC	1.64 ± 0.05	1.63 ± 0.03	1.64 ± 0.08	1.69 ± 0.02
LDL	0.46 ± 0.03	0.45 ± 0.02	0.44 ± 0.03	0.43 ± 0.01
HDL	0.92 ± 0.04	0.93 ± 0.02	0.9 ± 0.07	0.92 ± 0.02
TG	1.36 ± 0.11	1.35 ± 0.25	1.34 ± 0.23	1.42 ± 0.23
Week 2				
TC	1.59 ± 0.05	1.99 ± 0.08^{ax}	1.94 ± 0.12	1.91 ± 0.1
LDL	0.46 ± 0.02	0.77 ± 0.01^{ax}	0.76 ± 0.02^{x}	0.81 ± 0.05 ^{ax}
HDL	0.93 ± 0.03	0.89 ± 0.02	0.85 ± 0.04	0.86 ± 0.03
TG	1.33 ± 0.16	2.1 ± 0.16^{x}	2.11 ± 0.12^{x}	2.06 ± 0.18^{x}
Week 4				
TC	1.63 ± 0.03	2.09 ± 0.09^{axyz}	1.53 ± 0.16	1.6 ± 0.1
LDL	0.47 ± 0.02	0.81 ± 0.02^{axyz}	0.52 ± 0.07	0.6 ± 0.07
HDL	0.92 ± 0.04	0.81 ± 0.03	0.82 ± 0.04	0.84 ± 0.02
TG	1.41 ± 0.24	2.18 ± 0.21^{axyz}	1.3 ± 0.1b	1.15 ± 0.07^{b}
Week 6				
TC	1.56 ± 0.04	2.36 ± 0.06 ^{axyz}	1.47 ± 0.1	1.59 ± 0.13
LDL	0.46 ± 0.02	0.84 ± 0.01 ^{axyz}	0.33 ± 0.05	0.26 ± 0.02^{c}
HDL	0.89 ± 0.03	0.78 ± 0.04^{a}	0.81 ± 0.03	0.82 ± 0.04
TG	1.4 ± 0.25	2.66 ± 0.13^{axyz}	1.15 ± 0.38^{b}	0.83 ± 0.1^{b}
Week 8				
TC	1.55 ± 0.02	2.49 ± 0.06 ^{axyz}	1.52 ± 0.15	1.21 ± 0.14^{b}
LDL	0.46 ± 0.03	0.84 ± 0.01^{axyz}	0.27 ± 0.05^{b}	0.24 ± 0.04^{bc}
HDL	0.9 ± 0.03	0.86 ± 0.08	0.79 ± 0.04	0.82 ± 0.04
TG	1.37 ± 0.25	2.62 ± 0.12^{axy}	1.67 ± 0.21	1.22 ± 0.17^{b}

a, $p<0.05$ vs. week 0 for particular group within column
b, $p<0.05$ vs. week 2 for particular group within column
c, $p<0.05$ vs. week 4 for particular group within column
x, $p<0.05$ vs. control group, C, for particular week within row
y, $p<0.05$ vs. neem 50 mg/kg, N1, for particular week within row
z, $p<0.05$ vs. neem 300 mg/kg, N2, for particular week within row

4. Discussion

The rats fed with cholesterol-rich diet showed a steady rise in serum concentrations of total cholesterol as compared to rats given normal diet [10]. A simultaneous increase was observed in LDL and TG. HDL, however, showed a significant decrease ($p<0.05$) after six weeks of cholesterol feeding. When the ethanolic extracts of *Azadirachta indica*, a.k.a Neem, at the concentrations of 50 and 300 mg/kg body wt./day were administered to N1 & N2, TC, LDL and TG were not significantly affected.

It is most interesting to see that the serum CRP rose only in the cholesterol control group. Both 50 and 300 mg/kg of Neem extracts had lipid-lowering capabilities even during a continuous and prolonged feeding of dietary cholesterols. High CRP values seen in cholesterol group were also attenuated by Neem extracts.

Characteristics of the animal model used in the present study were similar to previous studies [11, 12]: animals fed a hypercholesterolemic diet showed higher serum cholesterol levels. Similar rise were seen in LDL-cholesterol and triglyceride levels of rats

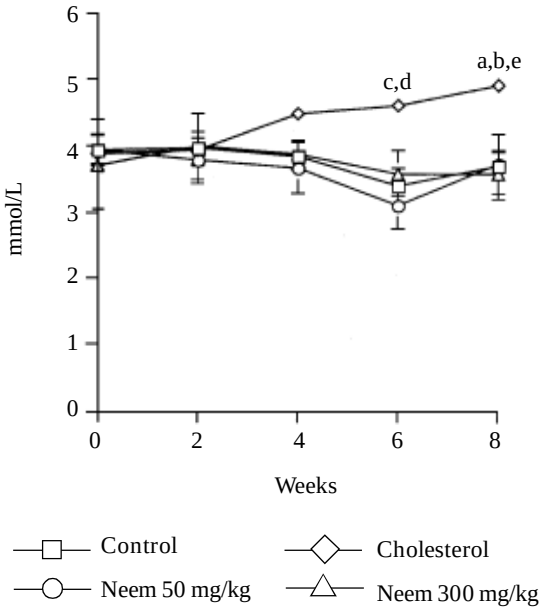

Fig. 2.
Changes in c-reactive protein concentrations in control and neem-treated rats

a, $p<0.05$ vs. week 0 for cholesterol control group (CC)
b, $p<0.05$ vs. week 2 for cholesterol control group (CC)
c, $p<0.05$ vs. control group at week 6
d, $p<0.05$ vs. neem 50 mg/kg at week 6
e, $p<0.05$ vs. all groups at week 8

from the CC group. Treatment with Neem extract successfully prevented dyslipidemia from occurring in separate sets of cholesterol-fed rats. The level of HDL, the 'good' cholesterol was also maintained within the normal range.

Even though this is still a preliminary study, it is the first documented and reported work regarding the anti-dyslipidemic activity of Neem. Neem is highly regarded among the Indian community, and has been used in the Ayurvedic medicine since 4500 years ago to cure many illnesses. The latest is the anti-ulcer properties of the bark of Neem tree [13]. The result of the present study is to add to its many other beneficial effects. One that is related includes its anti-inflammatory activity [5, 6]. Hypercholesterolemia if not treated may lead to atherosclerosis, a chronic, multifactorial, inflammatory disease characterized by the focal accumulation of inflammatory cells.

In fact, local inflammatory processes are present from the early stage and responsible for the formation of plaque. It is possible that in addition to Neem's anti-hypercholesterolemic activity that consequently reverse all the effects of hypercholesterolemia including inflammation and atherogenesis, its anti-inflammatory property could also play a role in suppressing this deleterious inflammatory process.

This anti-inflammatory activity of Neem is reflected in the serum concentrations of C-reactive protein (CRP) of Neem-treated rats. The rise in CRP seen in cholesterol-fed rats is diminished when given Neem extracts at 50 and 300 mg/kg body wt./day.

One of the theories regarding CRP suggested by Ferranti and Rifai (2002) [14] is that CRP is a marker of vascular inflammation that is released from atherosclerotic sites, a theory supported by the finding of decreased forearm vascular responsiveness in patients with increased CRP, and the inflammatory histology of unstable coronary plaques. Thus, when inflammatory processes are prevented it is possible that increased CRP level might also be diminished.

The present study is one of the few experimental studies that associate high cholesterol intake with increased CRP level. Recent investigations provide new aspects into the research field with regard to CRP. CRP has been regarded as a marker of inflammation; however, it has now become evident that CRP itself has a direct pro-inflammatory effect on vascular cells such as induction of adhesion molecules and chemokines [15]. New data suggest CRP in the presence of

serum mediates the uptake of LDL into macrophages, which then become foam cells [16] whereas Inoue *et al.* (2004) [17] suggest that CRP is a key molecule linking oxidative stress and inflammation which in the end leads to plaque instability. These findings see CRP as a culprit in atherogenesis.

Although this theory could not be verified by our findings, it was sufficient to suggest that CRP could have contributed to atherogenesis since high levels of CRP were seen in the later stages of feeding, a point of time where atherogenesis could have well developed. The CRP increments were also related to the rise in concentrations of serum lipids. CRP only increased significantly after two weeks of increased TC, LDL & TG.

Based on the results of our present investigation, Neem possesses potent cholesterol-lowering and anti-inflammatory properties which provide the scientific proof for the usage of this herb in traditional medicine. Future study must be performed to determine the mechanism of actions for both the anti-hypercholesterol and anti-inflammatory activities.

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