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THEOBROMINE ADMINISTRATION INHIBITS PLATELET AGGREGATION AND ELEVATES SERUM HDL-CHOLESTEROL IN HYPERLIPIDEMIC WISTAR RATS

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ABSTRACT

Pure theobromine was administered by oral gavage to hyperlipidemic Wistar rats weighing 100-250g for a four-day period. Serum lipid profiles, body weight changes and extent of platelet aggregation were assessed in these animals against appropriate controls. Theobromine administration in moderate (600mg/kg body weight) to high (700mg/kg body weight) doses, produced a significant ($P < 0.05$, and $P < 0.001$) dose and time dependent decrease in triglyceride, LDL-cholesterol and VLDL-cholesterol concentration and a significant ($P < 0.001$) increase in HDL-cholesterol level relative to controls. The results also indicate a significant ($P < 0.001$) increase in platelet count ratio in the theobromine treatment groups with values of 0.89 ± 0.08 and 0.99 ± 0.05 for the 600mg/kg and 700mg/kg theobromine dose regimens respectively, relative to the control value of 0.84 ± 0.11. The increase in the ratio indicates absence of aggregation; thus theobromine inhibits platelet aggregation and elevates plasma HDL-cholesterol levels in hyperlipidemic Wistar rats. The implications of these findings are discussed in relation to the therapeutic potentials of theobromine in the treatment of hyperlipidemia, and its ability to impede atherogenesis.

Key Words: Theobromine, hyperlipidemia, platelet-aggregation, HDL-cholesterol, atherogenesis.

INTRODUCTION

Theobromine is essentially a methylated xanthine, commonly known as 3, 7- dimethyl xanthine, prepared from the dried ripe seed of *Theobroma cacao* or made synthetically. Theobromine is known to be responsible in part for the stimulant action of cocoa and other beverages. (Southon and Buckingham, 1989; Eteng et al, 1997).

The members of the methyl xanthine family, viz. caffeine, theobromine and theophylline, are known to elicit pharmacological responses due to their alkylated acidic amine residues. (Kunibara and Tadokoro. 1992). In this wise, theobromine finds application in human medicine as a diuretic. At the therapeutic dose of 500mg, it is used in the treatment of cardiac oedema and angina pectoris. Theobromine is also used as a stabilizer in vitamin preparations. Although its use as a diuretic is rather limited nowadays due to the appearance of more efficacious drugs, yet theobromine analogues like pentoxyfylline. (Chang et al, 1993), lisofylline (Clark, 1996), suramin-theobromine complex (Gil et al, 1993), and even caffeine (BBC, report 1997) inhibit the growth of cancer cells, thus opening an exciting new horizon in cancer chemotherapy. There is thus a growing use of methyl xanthine alkaloids in chemotherapy. In a preliminary study with crude extracts of theobromine from cocoa bean seeds conducted in our laboratory, we observed that the administration of this extract elevated HDL-cholesterol levels but lowered other lipid components, while platelet aggregation was also inhibited in Wistar rats. In line with the current focus of researchers, we investigated the effect of theobromine on platelet aggregation and lipid levels in hyperlipidemic rats to further explore its pharmacological potentials.

MATERIALS AND METHODS

Animals

Forty albino rats of the Wistar strain consisting of both males and females were purchased from the disease-free stock of the Animal House, Biochemistry Department, University of Calabar, and reared on a popular commercial stock diet (Pfizer Livestock Feeds, Nigeria), until they were 120 days old and weighed 100-200g. These rats were then weighed and allocated on the basis of weight and litter origin to four groups (A, B, C, D) of ten animals each.

The rats were housed individually in specially designed perspex cages with a plastic bottom grid and a steel top (North Kent Plastic Cages Ltd., England) and kept under adequate ventilation at

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temperature and relative humidity of 26 ± 2°C and 46% respectively.

Induction of hyperlipidemia in rats

Rats in Groups B, C and D were placed on atherogenic diets compounded by blending boiled egg yolks with grower mash in the ratio of 2:3 by weight, and fed for 28 days, while control Group A was on the normal, non-atherogenic basal diet (grower mash) for the same period of time. The nutritional composition of the growers mash was protein (15% min), fats (3.5% min), fibre, (7.5% max), calcium (1.0% min), phosphorus (0.4% min), metabolisable energy (2400 Kcal/kg/ min). Food and water were supplied to all the rats ad libitum.

Administration of theobromine

Pure synthetic theobromine (3.7-dimethyl xanthine; melting point 351°C, UV absorption maximum 274nm and minimum 251nm; pH 10.6) was supplied by the British Drug House (BDH) Chemicals, Poole, England. It has identical physical characteristics to those of naturally occuring compounds in cocoa bean seeds. 22.5g of anhydrous theobromine were dissolved in 100ml of 1m sodium acetate solution to yield a unimolar theobromine stock solution of concentration 22.5g/100ml of sodium acetate. From this stock, both high (700mg/kg body weight) and low (600mg/kg body weight) doses were obtained by appropriate dilution and used for the study. At the end of the experimental feeding period, rats in control groups A and B were gavaged with 0.5ml of sodium acetate, respectively, while those of Groups C and D received low dose (600mg/kg body weight) and high dose (700mg/kd body weight) of theobromine, respectively. The oral administration was done daily for four days. The experimental design provide for two controls viz. control Group A and control Group B. This allowed for comparison of control group A with B to assess the extent to which lipid levels were elevated by the feeding of the atherogenic diet. The control Group B was compared with Groups C and D to assess the effect of theobromine on platelet aggregation ratio and lipid parameters. Twenty four hours after the last administration, the animals were suffocated in chloroform vapour, dissected and blood from each animal collected into two tubes, viz. tube A containing anticoagulant (0.72m EDTA) and tube B with no anticoagulant. Serum was separated from the blood sample in tube B by centrifugation at 800 revolutions per minutes for 15 min, after the sample had been allowed to stand for one hour for clotting to take place. The serum samples were stored at 4°C in a refrigerator and analyzed on the second day. The blood sample in tube A was immediately used for the determination of platelet aggregation ratio using the method of Wu and Hoak (1974).

Lipid profile analysis

Component lipids were estimated using enzymatic calorimetric diagnostic kits obtained from Randox Laboratories, Antrim, UK, in which the GPO-PAP method of Trinder (1969) was used for determination of serum triglycerides. The CHOP-PAP method of Richmond (1973) and Flegg (1973) was used for estimation of total cholesterol in serum, and the phosphotungstate precipitation method of Richmond (1973) was used for determination of HDL-cholesterol in serum.

The VLDL-cholesterol content of serum was determined according to Burnstein and Samaille's (1960) method. The serum triglyceride concentration was divided by the factor 5. This factor is based on the understanding that in fasting human subjects with triglyceride concentration of 400mg/dl the VLDL to total plasma triglyceride ratio is fixed relatively at 1:5. The LDL-cholesterol concentration was estimated as the difference between total cholesterol and the sum of HDL - cholesterol and VLDL - cholesterol. (Friedwald et al, 1972).

Determination of platelet count and platelet aggregation ratio

The method of Wu and Hoak (1974) was used. Briefly, 0.2ml of blood sample was pipetted into a first sample tube containing 3.8ml of buffered EDTA only with 1:2 dilution of blood obtained. Another 0.2ml of blood from the same animal was pipetted into a second tube containing 3.8ml buffered EDTA/ Formalin Solution. Both sample tubes were centrifuged at 200g for 8 minutes. Formalin fixes any platelet aggregates and causes it to precipitate during centrifugation. The platelets in the platelet samples obtained from both sample tubes after centrifigation were each counted using a light microscope (Olympus, UK) and a haemocytometer. After obtaining platelet counts, the ratio of platelet count in buffered EDTA/Formalin solution to the platelet count in buffered EDTA solution was estimated to give a quantitative detection of the extent of aggregation. There is no aggregation when the ratio approaches unity but aggregation takes place when the ratio is drastically reduced.

Statistics

The Student's t test was employed for statistical analysis and values of $P < 0.05$ were regarded as significant.

RESULTS

Table 1 presents the effect of atherogenic diets (egg yolk meal) fed for 28 days on lipid profile of Wistar rats and also the changes in lipid profile induced in Wistar rats with hyperlipidemia upon oral exposure to low and high doses of theobromine. By comparing the lipid profile data of animals in Group A (control I) with those of the experimental Group B (control 2), the extent to which lipid levels were elevated by the atherogenic diet fed for 28 days, were assessed. The mean ± SD values of serum TG (mmol/L), VLDL-cholesterol (mmol/L), total serum cholesterol (mmol/L), cholesterol (mmol/L) and LDL cholesterol (mmol/L) for Group A animals placed on the normal, non-atherogenic diet were 0.64 ± 0.09, 0.40 ± 0.04, 2.47 ± 0.29, 1.01 ± 0.11, and 1.05 ± 0.18, respectively. Those for Group B animals placed on atherogenic diet were 0.90 ± 0.11, 0.44 ± 0.05, 2.55 ± 0.30, 0.87 ± 0.08, and 1.17 ± 0.19, for, TG, VLDL -cholesterol, total serum cholesterol, HDL-cholesterol, and LDL cholesterol, respectively. The

Table 1: Effect of theobromine on serum lipid profile of experimental animals fed atherogenic (Groups B, C and D) and non-atherogenic diets (Group A) for 28 days.

	LIPID PARAMETERS (mmol/L)				
EXPERIMENTAL GROUP	TRIGLYCERIDE	VERY LOW DENSITY LIPOPROTEIN CHOLESTEROL	LOW DENSITY LIPOPROTEIN CHOLESTEROL	HIGH DENSITY LIPOPROTEIN CHOLESTEROL	TOTAL SERUM CHOLESTEROL
A (CONTROL 1) Rat chow + Sodium Acetate	0.64 ± 0.09	0.40 ± 0.04**	1.05 ± 0.18	1.01 ± 0.11	2.47 ± 0.29
B (CONTROL 2) Rat chow + Egg Yolk meal + Sodium acetate	0.90 ± 0.11	0.44 ± 0.05	1.17 ± 0.19	1.87 ± 0.08	2.55 ± 0.30
C Rat chow + Egg yolk meal + 600mg/kg (body weight) theobromine	0.46 ± 0.07**	0.22 ± 0.05*	0.48 ± 0.08**	1.10 ± 0.05*	1.79 ± 0.21
D Rat chow + Egg yolk meal + 700mg/kg (body weight) theobromine	0.39 ± 0.07**	0.17 ± 0.02*	0.60 ± 0.11**	1.30 ± 0.07*	2.07 ± 0.26

Results are presented as mean ± SD
* = Significantly different from Group B ($p<0.001$).
** = Significantly different from Group B ($p<0.05$).

results indicate that only HDL-cholesterol levels were decreased while all other lipid parameters were raised in the Wistar rats of Group B, although the increase was only statistically significant ($P < 0.05$) for VLDL cholesterol. Thus, except for HDL-cholesterol levels which were lowered, feeding of atherogenic diet induced hyperlipidemia in Wistar rats.

As also observed in Table 1, the oral administration of theobromine at 600mg/kg (Group C) and 700mg/kg (Group D) to Wistar rats with elevated serum lipids (hyperlipidemia) produced a dose dependant decrease in all other lipid parameters but significantly ($P < 0.001$) increased HDL-cholesterol levels. However, the decrease was statistically significant ($P < 0.05$) for TG, LDL and $P < 0.001$ for VLDL). The values of TG, VLDL-cholesterol, total cholesterol, HDL-cholesterol, and LDL-cholesterol for the control group B were 0.90 ± 0.11, 0.44 ± 0.05, 2.55 ± 0.30, 0.87 ± 0.08, and 1.17 ± 0.19 respectively, whereas those for the low dose theobromine treatment Group C were 0.46 ± 0.07, 0.22 ± 0.05, 1.79 ± 0.21, 1.10 ± 0.05, and 0.48 ± 0.08. Those for the high dose theobromine treatment were 0.39 ± 0.07, 0.17 ± 0.02, 2.07 ± 0.26, 1.30 ± 0.07, and 0.60 ± 0.11, all in mmol/L, respectively. Table 2 presents the mean ± SD values of platelet count in EDTA solution only and computed platelet aggregation ratio in control Group B and experimental animal group C treated with 600mg/kg theobromine and Group D which received 700mg/kg theobromine. The values of platelet count ratio for control Group B, Group C and Group D were 0.84 ± 0.11, 0.88 ± 0.08, and 0.98 ± 0.05, respectively. The results indicate a highly significant ($P < 0.001$) increase in the platelet count ratio in both test groups administered with theobromine as reflected in the value of 0.88 ± 0.08, for Group C, and 0.98 ± 0.05 for Group D compared to control Group B with a ratio of 0.85 ± 0.16.

DISCUSSION

The causal role of elevated serum cholesterol (hypercholesterolemia) in the genesis of atherosclerosis and its clinical sequelae particularly ischemic heart disease, is now well established in many population groups all over the world, (Aronow and Ahn, 1994). Atherogenesis leading to myocardial infarction and its associated consequences like heart failure, remains the leading cause of death in the western world (Onunu, 1996). Considerable effort has been made in recent times, through the development of drugs that lower serum cholesterol levels in patients with hypercholesterolemia and through dietary control, at the reduction of saturated fats in the diet. The chemotherapeutic potentials of the methylxanthines and their analogues are currently being exploited in the treatment of diseases such as

cardiac oedema, angina pectoris and cancer (Rall, 1980; BBC Report, 1997 unpublished).

In line with this current focus of research, the present study assessed the induction of hyperlipidemia upon feeding atherogenic, egg yolk-based meals for 28 days to Wistar rats. An elevation of lipids was observed which agrees with the reports of Woosley et al (1973), White, et al (1978) and Gaw and Shepherd (1997). It is also established that dietary factors produce changes in body composition, physiology and biochemical functions (Erdman and Fordyce. 1989; Carroll, 1991). The effects of theobromine administration on the lipid profile and platelet aggregation status of hyperlipidemic rats were also assessed. The results indicate a significant decrease in triglycerides, LDL- and VLDL-cholesterol concentrations, but a highly significant increase in HDL-cholesterol, in hyperlipidemic rats at both high and low theobromine dose regimens. Platelet aggregation was also inhibited. The probable explanation for the observed results on lipid profile, may centre on the perturbation in lipid metabolism caused by the methylxanthine through the inhibition of phosphodiesterase breakdown of cAMP. The accumulating cAMP activates lipase and phosphorylase enzymes which act on parameters of lipolysis and glycolysis (Granner, 1990).

The significant increase in HDL-cholesterol levels with the significant reduction in other lipid parameters unequivocally suggest that theobromine plays a protective role of against atherogenesis. Many factors may explain the observed inhibition of platelet aggregation by methylxanthine. According to Onunu (1996), HDL inhibits platelet aggregation and stimulates arterial prostacyclin production. Since theobromine induces a statistically significant increase in HDL-cholesterol, it explains why it acts indirectly to inhibit platelet aggregation. Linder and Good (1982) have explained that the elevation of cAMP caused by high levels of arachidonic acid inhibits platelet aggregation. Theobromine, by inhibiting phospho-diesterase, similarly causes accumulation of cAMP, which in turn inhibits platelet aggregation. This provides a second mechanism by which the purine alkaloid may inhibit platelet aggregation.

The inhibition of platelet aggregation by theobromine is consistent with earlier reports by Ardlie et al (1967) for the xanthine. In general, platelets participate in phagocytosis, inflammation, nourishment of the vessel lining and formation of prostaglandins. Immunologically, they contribute to organ rejection, metastasis, complement activation and thrombosis (Suohiro et al, 1982). Aggregation of platelets at sites of vascular injury is one of the physiological steps in the pathway leading to atherosclerosis according to the modified Virchow's insudative theory of atherogenesis (Faggiato and Rose, 1984). The ability of theobromine in both low and high doses to significantly reduce all other lipid parameters but elevate HDL-cholesterol in hyperlipidemic rats, coupled with the inhibition of platelet aggregation, affirms that theobromine impedes the atherogenic process, and can be applied in the treatment of hyperlipidemia and associated clinical sequelae leading to cardiac failure.

CONCLUSION

Taking together the findings of this study, that theobromine administration in both moderate to high doses inhibits platelet aggregation, elevates HDL-cholesterol levels but lowers total serum cholesterol, LDL-cholesterol, VLDL-cholesterol and triglyceride levels in serum of hyperlipidemic rats, we conclude that since platelet aggregation is

Table 2: Platelet count in EDTA/Formalin, EDTA solution only and computed platelet count ratio in the control and experimental groups treated with theobromine.

	PARAMETERS		
EXPERIMENTAL GROUP	PLATELET COUNT IN EDTA/FORMALIN SOLUTION x 10^3/mm^3	PLATELET COUNT IN EDTA/SOLUTION ONLY x 10^3/mm^3	PLATELET AGGREGATION RATIO
B (CONTROL 2) Rat chow + Egg Yolk meal + Sodium acetate	267.01 ± 49.00	315.03 ± 17.14	0.84 ± 0.11
C Rat chow + Egg yolk meal + 600mg/kg (body weight) theobromine	184.09 ± 33.11	212.07 ± 40.20	0.87 ± 0.08*
D Rat chow + Egg yolk meal + 700mg/kg (body weight) theobromine	239.31 ± 32.00	241.22 ± 31.34	0.99 ± 0.05*

Results are presented as mean ± SD

* = Significantly different from control ($p<0.001$).

inhibited by theobromine, coupled with the elevation of serum HDL-cholesterol, which plays a protective role against atherogenesis, theobromine thus impedes the atherogenic process. By also significantly reducing total serum cholesterol, LDL-cholesterol, VLDL cholesterol and triglyceride levels in hyperlipidemic rats, theobromine holds promising therapeutic potentials in the treatment of hyperlipidemia.

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