INFLUENCE OF DIETARY FATS ON ATHEROSCLEROSIS, COAGULATION AND PLATELET PHOSPHOLIPIDS IN RABBITS

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SUMMARY

Male rabbits were fed for six months diets comprising cholesterol (0.1%) and either butter alone (10%) or butter (5%) plus cacao butter, coconut oil, olive oil, or corn oil (4.5%). These fats could be classified according to their atherogenicity as follows, in decreasing order: butter, olive oil, coconut oil, cacao butter, and corn oil. The severity of the atherosclerotic lesions was correlated with the plasma cholesterol.

By contrast, concerning their effect on the recalcification plasma (platelet-rich) clotting time (PCT), these fats could be classified in the following way: cacao butter, butter, coconut oil, olive oil and corn oil. The values of the PCT were significantly correlated with the ratio stearic/linoleic acid in the dietary fats, the clotting activity and the fatty acid composition of the platelet phospholipid fractions examined (phosphatidyl serine | phosphatidyl inositol), but not with the concentration of these fractions in platelets. Butter was the only fat able to induce severe alterations at the same time in coagulation (presumably through an increase in the activity of certain platelet phospholipids), lipemia and arterial wall morphology.

RÉSUMÉ

Des lapins mâles ont été nourris pendant six mois de régimes contenant du cholestérol (0.1%) et soit du beurre seul (10%), soit du beurre (5%) auquel on avait ajouté du beurre de cacao, de l’huile de coco, de l’huile d’olive ou de l’huile de maïs (4.5%). Selon leur caractère athérogène, on a pu classifier ces graisses de la façon suivante, par ordre décroissant: beurre, huile d’olive, huile de coco, beurre de...
cacao et huile de maïs. On a trouvé une corrélation significative entre la sévérité des lésions d’athérosclérose et le cholestérol plasmatique.

Par contre, en ce qui concerne leur effet sur le temps de récalcification du plasma (riche en plaquettes) (PCT), on a pu classer ces graisses par ordre décroissant de la façon suivante: beurre de cacao, beurre, huile de coco, huile d’olive, huile de maïs. On a trouvé une corrélation significative entre les valeurs du PCT et le rapport acide stéarique/acide linoléique des graisses du régime, l’activité coagulante des phospholipides plaquettaires examinés (phosphatidyl serine + phosphatidyl inositol) de même qu’avec leur composition en acides gras. Aucune relation n’a pu être montrée entre la concentration plaquettaire de ces phospholipides et leur activité coagulante. Le beurre était la seule graisse capable d’induire en même temps une hypercoagulabilité (in vitro) (par l’intérimediaire, semble-t-il, d’une augmentation de l’activité de certains phospholipides plaquettaires) et une hypercholestérolémie marquée, ainsi que des lésions sévères d’athérosclérose.

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**Key words:**  *Atherosclerosis – Dietary fats – Fatty acids – Hypercoagulability – Platelet phospholipids*

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**INTRODUCTION**

Several studies have indicated that the consumption of saturated fats contributes to the development of coronary heart disease in the human population\(^1^\)\(^-\)\(^3\). Since such heart disease is associated with two main conditions, namely atherosclerosis and thrombosis, many studies have been performed to determine the effect of fats on these two conditions. While it has been repeatedly shown that saturated fats increase the severity of atherosclerosis in animals\(^4^\)\(^-\)\(^7\) it is not yet clear whether these same fats are able to induce a state of hypercoagulability, which would predispose for thrombosis\(^8\).

In recent experiments in rats, rabbits and man\(^9\), we observed that *in vitro* hypercoagulability, in relation to hyperlipemia, was mostly due to an enhanced activity of platelet factor 3. We had previously reported\(^10^,\)\(^11\) that the effects of dietary saturated fats on thrombosis and coagulation in the rat were mostly due to stearic acid.

In order to further characterize the effect of saturated lipids, on atherosclerosis and coagulation, we have conducted investigations in the rabbit concerning the influence of various dietary fats on the severity of atherosclerosis, and on the clotting changes they induce in relation to certain platelet phospholipids known to be involved in factor 3 activity. Results of this study are reported hereafter.
MATERIAL AND METHODS

Animals

The 50 New-Zealand male rabbits (initial body weight 1,200 to 1,500 g) utilized in this study were purchased from a local breeder. For 6 months, the animals were fed, ad libitum, a commercial diet (Purina rabbit chow checkers, Ralston Purina Co. of Canada) (89.9%) to which was added one of the five following fat mixtures (in weight %).

- Group 1 = butter 10, cholesterol 0.1
- Group 2 = butter 5, cacao butter 4.5, water 0.5, cholesterol 0.1
- Group 3 = butter 5, coconut oil 4.5, water 0.5, cholesterol 0.1
- Group 4 = butter 5, olive oil 4.5, water 0.5, cholesterol 0.1
- Group 5 = butter 5, corn oil 4.5, water 0.5, cholesterol 0.1

Blood removal

After overnight fasting, blood was obtained from the jugular vein, under light ether anesthesia, the first drops of blood being discarded. After blood removal, the siliconized syringe containing the appropriate anticoagulant was immediately and gently inverted three times. The blood was then transferred into silicone-coated tubes.

Clotting tests

Unless otherwise stated, all the glassware was coated with silicone (General Electric Dry-Film, SC-87, in carbon tetrachloride). However, in each experimental series, the final clotting determinations were performed, in duplicate, in plastic (polycarbonate No. 2804, International Equipment Co.). In addition, when evaluating the phospholipid clotting activity, a duplicate of final determinations was also performed in disposable, non-siliconized, glass tubes (10 × 75 mm).

Plasma clotting time (PCT)

This test has been described in detail previously. Blood (1 ml) was collected in a 2 ml siliconized syringe containing 0.1 ml of 3.8% sodium citrate. The anticoagulated blood was stored at 30 °C and centrifuged (6 min, 700 g) at room temperature.

The platelet rich plasma (PRP) was then removed with a silicone-coated Pasteur pipet and stored again at 30 °C for periods up to 2 h.

In the present series, 0.1 ml of plasma was placed in the polycarbonate tube and incubated for 10 min at 37 °C. The test was started by blowing 0.2 ml of CaCl₂ (0.01 M) into the plasma. The platelet count in the PRP was determined in four animals per group.

Phospholipids clotting time ((PS + PI) CT)

For this test, a standard platelet-poor plasma has first to be prepared. From a 3 kg rabbit fed the commercial diet only, 50 ml of blood were removed in siliconized syringes containing citrate as the anticoagulant (1 vol. of sodium citrate for 9 vol. of
blood), and centrifuged at 30 °C (30 min, 2000 g). The platelet-poor plasma was then collected, distributed in small siliconized containers, and frozen. Prior to testing, these plasma samples were thawed and stored for 30 min at 30 °C, at which temperature they could be kept for 2 h without changes in the results. When recalcified without addition of platelets or phospholipids, each of these samples from the same pool of plasma, presented a clotting time ranging from 450 to 500 sec.

The phospholipid fractions (phosphatidyl serine (PS) + phosphatidyl inositol (PI)) in quantity corresponding to 2 × 10⁹ platelets, separated and purified as described below, were stored in a chloroform–methanol (1:1) mixture. A few minutes before determination of the clotting time, this mixture was evaporated to dryness at 35–37 °C under nitrogen flow and suspended, by sonication for 15 sec, in 1 ml of incomplete Tyrode’s solution containing no Ca and Mg (NaCl 8.7; KCl 0.2; NaHCO₃ 0.8; dextrose 1.0; NaH₂PO₄•H₂O 0.05; in grams and distilled water to 1,000 ml), the pH being adjusted to 7.4 with 1 N HCl immediately before use.

This suspension was then transferred into a small plastic tube. After a strong agitation, 0.1 ml of the suspension was added to 0.1 ml of the standard platelet-poor plasma in a polycarbonate or glass tube. The phospholipid suspension and the plasma were gently mixed by rotating the tube and then incubated for 10 min at 37 °C.

The clotting test was performed by recalcification of the plasma with 0.1 ml of 0.02 M CaCl₂.

Lipid extracts

Blood (50 ml) anticoagulated with EDTA (EDTA, 1.6 g; NaCl, 0.66 g; dextrose, 0.1 g; distilled water to 100 ml) (9 vol. of blood for 1 vol. of EDTA solution), was kept and centrifuged (8 min, 220 g) at room temperature. The platelet-rich plasma was removed and centrifuged (18 min, 1,000 g) for collection of platelets. The platelets were washed twice in incomplete Tyrode’s solution prepared as mentioned above except that the pH was 6.6. At the last washing, the platelets were counted with a Coulter Counter (Coulter Electronics Inc.) and then resuspended in incomplete Tyrode’s solution (pH = 7.4) at a concentration of 6 to 8 × 10⁶ per mm³, in a total volume of 1 ml.

Lipid extraction was performed according to the technique of Folch et al.¹³ by adding 25 ml of a chloroform–methanol (2:1) mixture to the platelet suspension and strongly agitating the container (tightly closed 50 ml centrifuge tube) for 1 min. Preliminary experiments had indicated that previous disruption of the platelets by sonication or freezing and thawing does not improve platelet lipid extraction as performed under the conditions described above.

After filtration, 5.2 ml of 0.73 M NaCl was added to the mixture, which was agitated for 20 sec. After removal of the upper layer before and after centrifugation (15 min, 500 g), the chloroform extract was evaporated to dryness at 35 °C with nitrogen. The lipids were again dissolved in 20 µl of a 2:1 chloroform–methanol mixture.

Thin-layer chromatography

Separation of the various lipid fractions was performed by thin-layer chromato-
graphy in two dimensions, according to the technique of Bunn et al., the only modification being the use of Silica Gel HF-254 (E. Merck, Darmstadt, Germany) instead of Silica Gel G, but still in borate buffer (pH = 8). The plates were prepared a few hours before utilization by applying a 0.35 mm layer of silica gel, which was then dried for 90 min at 60-80 °C and 60 min at 110-120 °C.

All procedures were carried out under highly purified nitrogen (Nitrogen U, Liquid Air of Canada) to avoid oxidation.

For each separation, the platelet lipids were applied to an experimental and a control plate, the migration being performed at the same time in the same chamber jar. After the first migration to an approximate height of 13 cm, the plates were dried for 15 min under nitrogen flow. On the control plate, the spots corresponding to the various fractions (previously identified by comparison with the migration of phospholipid standards) were localized by immersing the plate in a closed-chamber jar containing iodine crystals. The spots corresponding to the PS and PI fractions on the experimental plate were then detected by transparency, after superimposing this plate on the control plate. To verify the localization of the PS and PI spots, the experimental plate was also examined under ultra-violet illumination. Subsequently, the silica gel of the PS + PI spots was removed with a spatula and collected in a 50 ml beaker containing 5 ml of a chloroform–methanol (1:1) mixture. After filtration, the powder was washed with 2 ml of methanol and 2 ml of chloroform–methanol (1:1). Finally, to verify that the totality of the spots containing the fractions PS + PI had been removed, the experimental plate was immersed in the jar with iodine vapours.

Thus, the phospholipid extracts were divided into three parts: one for clotting tests performed immediately after the separation, one for phosphorus determination, and one for fatty acid analysis performed a few days later on samples stored at 4 °C under nitrogen.

Gas-liquid chromatography.

For fatty acid analysis, the methyl esters of the phospholipid fractions or of the lipid extract (dietary fat) were prepared according to a technique adapted from that of Nelson and Freeman. The chloroform–methanol extract of the phospholipid fraction was transferred into a 30 ml test tube that could be closed by a glass bead. After evaporation to dryness by nitrogen, 8 ml of 2% solution (by volume) of H2SO4 in methanol was added.

The tube was then closed by a glass bead and heated at 72 °C for approximately 2 h (until the solution was reduced to 1 ml). After removal of the tube from the heat, 4 ml of distilled water and 6 ml of petroleum ether (30-60° boiling range) were added successively. The mixture was then strongly agitated for 2 min, and the ether phase was collected and evaporated to dryness by nitrogen. The esters were dissolved in approximately 15 μl of heptane (chromato-quality reagent), and were then ready to be injected (4 μl) into the column.

For fatty acid analysis, a Hewlett-Packard gas chromatograph (model 402) was utilized, with helium (35 ml/min) as carrier gas and temperature programming
(140–200 °C, 4 °C/min). The two glass columns (6 feet) were packed with 6% EGS absorbed on Chromosorb (W, ADW–DMCQ, 80/100 mesh) (Chromatographic Specialities, Brockville, Ont., Canada).

Phosphorus determination
Phosphorus was determined according to the technique of Parker and Peterson\textsuperscript{16}. However, the ANSA reagent utilized was the Fiske and Subbarow reducer, at the concentration given by the supplier (Sigma Chemical Co., St. Louis, Mo.). The colorimetric determination was performed on a Coleman spectrophotometer (Hitachi 101) at 820 μ. The amount of phosphorus was given by comparison with a standard curve, verified from time to time.

Cholesterol and triglyceride analysis
These determinations in plasma were performed by the Technicon automated techniques.

Evaluation of lesions
After blood removal, rabbits were killed with chloroform. The surfaces of the heart and the aorta were examined with a binocular microscope, the aorta having been opened longitudinally from the aortic valve to the iliac arteries. The severity of lesions of the ascending, thoracic and abdominal parts of the aorta was assessed separately, according to the following gradation: 0, no lesions; 1, one to several white-yellowish raised fatty streaks; 2, 3, 4 involving respectively a quarter, a half, or the entire intimal surface. The final data reported in Fig. 1 are the means of the readings in the three parts of the aorta. The macroscopic reading was verified by routine histologic examination.

Statistical analysis
The correlation coefficients and their significance ($t$-test) were calculated according to Snedecor and Cochran\textsuperscript{17}.

RESULTS

As shown in Fig. 1, the most severe lesions of atherosclerosis were noted in the animals fed butter or olive oil. Histologically, the lesions in these two groups were characterized by a marked intima thickening filled with lipids and cholesterol crystals and covered by a fibrous cap rich in spindle cells. The least severe lesions were noted in cacao butter and corn oil fed rabbits. The cholesterolemia tended to present a pattern similar to the severity of the atherosclerotic lesions. For each animal the individual values of the plasma cholesterol were correlated with the severity of atherosclerosis: the coefficient obtained was $r = 0.54$, a highly significant figure ($P < 0.001$). In contrast to this, no significant correlation could be found between plasma triglycerides and the severity of atherosclerosis.
Fig. 1. Influence of dietary fats on atherosclerosis, lipemia, and coagulation in rabbits. Animals were fed for 6 months a commercial diet to which was added 0.1% cholesterol and one of the fats listed in the figure (butter alone (10%) or butter (5%) + cacao butter, coconut oil, olive oil, or corn oil (4.5%)). PCT: recalcification plasma clotting time. The percentages of dietary fatty acids are expressed by the ratio of the saturated/unsaturated (sat/unsat) and that of stearic/linoleic acid.

Fig. 2. Relationship between PCT and platelet phospholipid fractions PS + PI in rabbits fed the five different fats. (PS + PI) CT: clotting activity of these fractions resuspended in a standard platelet-poor plasma. The PS + PI concentration is expressed in μg per 2 × 10⁹ platelets.
As regards the PCT, no relationship could be established with the plasma cholesterol. However the mean PCT for each group was inversely correlated \((r = -0.87)\) with the ratio stearic/linoleic acid in the diet of each of these groups. In the four animals of each group in which it was determined, there was no significant difference in the platelet count between the groups (range 360 to \(440 \times 10^3\) platelets/mm\(^3\)).

In Fig. 2 are reported the clotting activity, fatty acid composition, and concentration of the platelet fractions PS + PI as compared to the PCT in the five groups studied. It can be noted that the clotting activity \((PS + PI) CT\) of the phospholipid fractions studied appears to be related to the PCT. For each animal, the values of the \((PS + PI) CT\) were significantly correlated \((P < 0.001)\) with the values of the PCT when the clotting activity of the phospholipids was determined either in plastic \((r = 0.78)\) or in glass \((r = 0.71)\).

The clotting activity of the PS + PI fractions was also correlated \((r = -0.53, P < 0.01)\) with their fatty acid composition (the ratio \(18:0 + 18:1/18:2 + 20:4\)) but not with the concentration of these fractions in platelets.

**DISCUSSION**

The results obtained here regarding plasma cholesterol and atherosclerosis generally agree with those reported by previous investigators, in that butter fat is highly hypercholesterolemic and atherogenic. However, a somewhat surprising finding was that, under these conditions, olive oil was more hypercholesterolemic and atherogenic than coconut oil, a highly saturated fat. This might be due to the fact that the fat fed was not pure coconut oil or olive oil, but a mixture of coconut oil (4.5\%) (or of the other fats) added to butter (5\%). It has already been reported that in rats\(^{18}\) interactions take place between dietary fatty acids that might change the specific effect of some fatty acids.

In contrast to the plasma cholesterol values and the severity of atherosclerosis, which could not be correlated with any ratio of the dietary fatty acids, the recalcification plasma clotting time (PCT) was correlated with the ratio stearic/linoleic acid in the diet, a result identical to that obtained in our recent studies in rats\(^{19}\). It is also concordant with our earlier findings which indicated that dietary stearic acid might be the most thrombogenic dietary fatty acid in rat\(^{10,11}\). Of interest is the observation that, among the dietary fats tested, butter is the only fat that has marked effects, at the same time, on plasma cholesterol, atherosclerosis, and coagulation.

Platelet factor 3 appears to be a lipoprotein of which the lipid moiety is a phospholipid, or phospholipid fractions of which the most active is PS, a result we confirmed recently\(^{20}\). However PI, containing some amounts of PS, has been found to be also very active\(^{21}\). The marked correlation seen here between the PCT and the clotting activity of the fractions PS + PI indicates that the *in vitro* hypercoagulability observed in the groups fed cacao butter and butter might be due to the enhanced activity of platelet factor 3, particularly since we have previously reported that an increase in
the activity or concentration of plasmatic clotting factors could not explain the \textit{in vitro} hypercoagulability induced by feeding butter to rabbits and rats\(^9\).

The increase in the activity of the platelet phospholipids does not appear to be due to an increase in the concentrations of these fractions, but might result from changes in their fatty acid composition, namely, an increase in stearic and oleic acids at the expense of the polyunsaturated acids. An increase in platelet oleic acid in response to stearic acid feeding is not unexpected, since it has been reported that, when administered to animals, stearic acid is rapidly transformed into oleic acid by the liver and other tissues\(^22\).

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Erratum


In the diagram of Fig. 1 on page 86 one arrow is missing. The diagram should appear as follows:

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High fat diet  hyperlipoproteinemia  GAG synthesis
            glucose uptake
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