

# Effects of low-fat milk enriched with phytosterols on plasma cholesterol concentrations and hemorheological parameters of Wistar rats

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**Abstract.** Clinical and experimental studies have shown that the use of phytosterol esters as a food ingredient reduces the plasma concentrations of cholesterol and LDL cholesterol, not affecting the HDL cholesterol levels. Based on the use of phytosterols as a food ingredient, we have conducted a 30-day feeding study with Wistar rats, drinking low-fat milk containing phytosterols, in order to evaluate the plasma cholesterol concentrations and the hemorheological parameters. Throughout the study, clinical observations, body weights and food and milk consumption were measured and at the end of the feeding period, blood samples were collected for biochemical and hemorheological determinations. There were no clinical changes, alterations in growth, food or milk consumption. In the plasma cholesterol and HDL concentrations there were no significant differences, but LDL levels decreased about 70%. In the hemorheological parameters, significant changes were observed in plasma viscosity and in membrane fluidity in all experimental groups. The blood viscosity and the erythrocyte deformability show significant improvements with the ingestion of the phytosterols enriched milk. With these results we conclude that phytosterols maintain their cholesterol lowering properties when incorporated in milk and can be considered a hypolipemic food component.

Keywords: Phytosterols, cholesterol, LDL-cholesterol, HDL-cholesterol, milk, hemorheology

## 1. Introduction

Phytosterols (PE), or plant sterols are not produced by animals or human body, they are natural substances usually found in nuts, vegetable oils, corn, rice and some other plants present in the human diet and they are structurally similar to cholesterol. When absorbed, phytosterols displace cholesterol from intestinal micelles, reducing the pool of absorbable cholesterol.

Studies on the efficacy of plant sterols in reducing the plasma cholesterol date back to the 1950s. It is now known that phytosterols effectively reduce LDL-cholesterol when given as supplements, and as smaller amounts in foods. Isolated VLDL and LDL added to plasma *in vitro* cause a dose-dependent and exponential rise in blood viscosity [1].

In more recent studies, the esterification of sterols has allowed the use of the phytosterols as a food ingredient, resulting in a food component to reduce the blood cholesterol and a number of studies have demonstrated the efficacy of these components in a variety of low fat foods such as bread, cereal and yogurt [3]. With phytosterols as functional food, we have conducted a 30-day feeding study in Wistar rats,

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using low-fat milk containing phytosterols, in order to evaluate the plasma cholesterol concentrations and the hemorheological parameters.

## 2. Materials and methods

The animals used in this study received human care in accordance with the Directive of the European Community n°86/609/CEE. Groups of 10 Wistar male rats (HsdBrlHan:Wist, Harlan Iberica) with a average weight of  $223.67 \pm 38.18$  g were used. After an adaptation period of one-week, the rats were housed singly and divided in 5 groups: Group 1 – low-fat milk with 0.2 g phytosterols/dl; Group 2 – low-fat milk with 0.3 g phytosterols/dl; Group 3 – low-fat milk with 0.4 g/dl of phytosterols; Placebo – low-fat milk without phytosterols and Control – water. Clinical observations, body weights and milk consumption were daily measured during the 30 days period of study.

After the drinking period, the animals were anesthetized for blood collection. Anesthesia was achieved with intraperitoneally urethane (Sigma) 1.5 g/kg body and intramuscular ketamine (Pfizer, Parke Davis) 50 mg/kg. Body temperature was maintained between 35–37°C with an auto-regulated heating platform. The left carotid artery was cannulated with polyethylene tubing and blood samples were collected.

### 2.1. Biochemical parameters

The plasma pH, sodium, potassium and chloride concentrations were determined with an ABL505 electrode system from Radiometer (Copenhagen, Denmark). Hemoglobin was measured with the Osm3 hemoximeter from Radiometer and red blood cell count, total white blood cell counts, and hematocrit were determined with a Cell Dyn 1600 hemocytometer (Abbott, Abbot Park, IL). Total cholesterol, LDL-Cholesterol and HDL-Cholesterol plasma concentration were determined using enzymatic-colorimetric tests (Spinreact, SA, Spain).

### 2.2. Hemorheological parameters

Blood samples anticoagulated with K<sub>3</sub>EDTA were centrifuged at 3000 rpm for 10 minute, and the resulting plasma was collected for the determination of plasma viscosity with the Harkness method [7]. Whole blood viscosity (WBV) was determined in a Brookfield digital viscometer model LVTDV II cp., using native blood aliquots submitted at low ( $22.5 \text{ s}^{-1}$ ) and high ( $225 \text{ s}^{-1}$ ) shear stress forces.

Membrane lipid fluidity was determined by measuring fluorescence anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) probe for hydrophobic zone of membrane, 1,4-(trimethyl)-phenyl-6-phenylhexa-1,3,5-triene (TMA-DPH) and 4-heptadecyl-7-hydroxicoumarin (HC) for the external region of the membrane. The fluorescence anisotropy was measured using a spectrofluorometer Hitachi F3000 (Hitachi, Japan), according to the method described by Shirilo et al. (1981) [8]. Erythrocyte deformability was determined using the Rheodyn SSD laser diffractometer from Myrenne (Roetgen, Germany).

## 3. Results

### 3.1. Body weights and milk consumption

There were no statistically significant differences in mean body weights observed for any of the experimental groups *versus* the control group. There was no effect on the food consumption.

Table 1

Hematological and biochemical parameters (mean  $\pm$  standard deviation) determined after a 30-day feeding period with low-fat milk

	Experimental groups				
	Control	Placebo	Group 1	Group 2	Group 3
Initial weight (g)	306 $\pm$ 58	364 $\pm$ 43	310 $\pm$ 17	295 $\pm$ 36	291 $\pm$ 24
Mean consumption (ml/rat/day)	30 $\pm$ 2	35 $\pm$ 6	39 $\pm$ 19	34 $\pm$ 19	34 $\pm$ 1
Hemoglobin (g/dl)	16.0 $\pm$ 1.1	17.2 $\pm$ 1.2	16.5 $\pm$ 1.0	15.9 $\pm$ 1.0	15.9 $\pm$ 1.0
Hematocrit (%)	52 $\pm$ 12	53 $\pm$ 15	49.3 $\pm$ 2.7	46 $\pm$ 2.0	47.2 $\pm$ 1.3
Red blood cell count ( $\times 10^{12}/l$ )	8.00 $\pm$ 0.91	7.03 $\pm$ 2.23	7.79 $\pm$ 1.39	8.31 $\pm$ 0.50	7.76 $\pm$ 0.90
White blood cells ( $\times 10^9/l$ )	4.15 $\pm$ 2.09	5.15 $\pm$ 1.48	4.53 $\pm$ 1.59	4.99 $\pm$ 0.67	4.79 $\pm$ 1.78
pH	7.35 $\pm$ 0.07	7.38 $\pm$ 0.04	7.41 $\pm$ 0.04	7.41 $\pm$ 0.03	7.42 $\pm$ 0.04
Sodium (mmol/l)	139.0 $\pm$ 1.0	134.88 $\pm$ 1.89 <sup>a</sup>	135.5 $\pm$ 2.6 <sup>a</sup>	135.5 $\pm$ 3.2 <sup>a</sup>	136.9 $\pm$ 2.6 <sup>a</sup>
Potassium (mmol/l)	3.50 $\pm$ 0.48	3.54 $\pm$ 0.23	3.74 $\pm$ 0.4 <sup>b</sup>	3.46 $\pm$ 0.32	3.89 $\pm$ 0.48 <sup>c</sup>
Calcium (mmol/l)	1.15 $\pm$ 0.05	1.13 $\pm$ 0.04	1.15 $\pm$ 0.06	1.16 $\pm$ 0.06	1.13 $\pm$ 0.07

<sup>a</sup>Statistically significant difference from the control group  $p < 0.001$ ; <sup>b</sup>statistically significant difference from the control group  $p < 0.007$ ; <sup>c</sup>statistically significant difference from the control group  $p < 0.003$ .

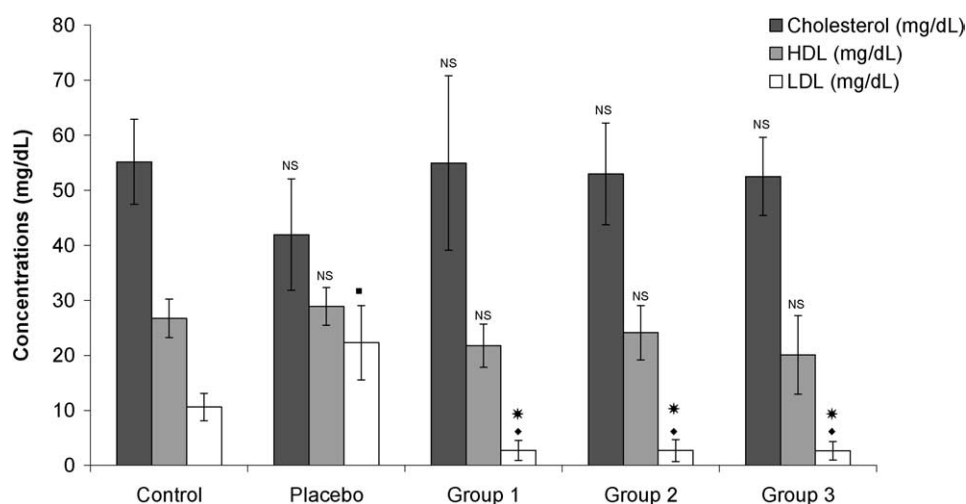


Fig. 1. Cholesterol, HDL and LDL plasma concentrations in Wistar rats after the ingestion of milk supplied with different phytosterols concentrations during a 30-day period. No significant (NS) differences were found between the control and the other experimental groups. The LDL levels decrease significantly comparing both with the control or the placebo group. There was an increase in the LDL plasma levels after the ingestion of milk with no phytosterols addition ( $*p < 0.0007$ ). Comparing to the control group there were significant decreases in the groups I, II and III ( $*p < 0.0001$ ). The differences between the placebo and the other experimental groups were also statistically significant,  $*p < 0.00001$ .

### 3.2. Biochemical parameters

The haematology parameters don't show statistically significant differences in any of the experimental groups. The variations obtained in sodium and potassium levels, although with statistical significance, were considered to have no biological significance (Table 1).

Analysing the cholesterol levels obtained in the experimental groups in study, we can see that there is no statistically significant difference between the control and the remaining groups. In the HDL-cholesterol plasmatic levels there are no statistically significant changes from the control group. The

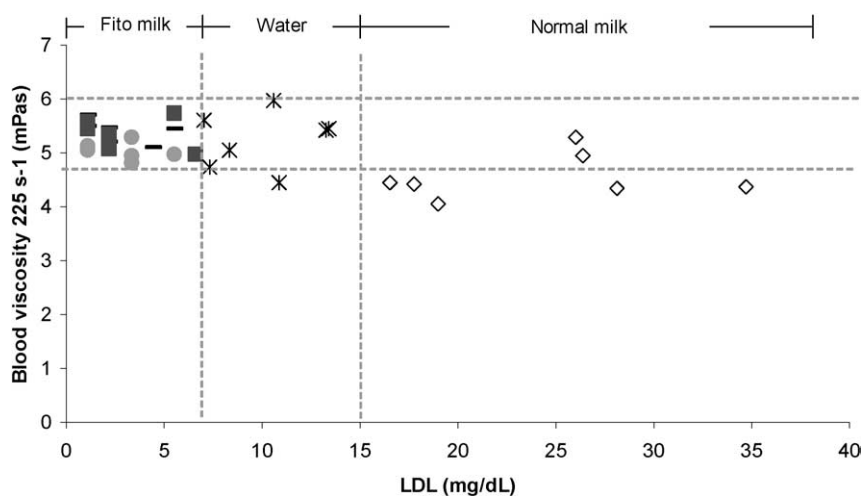


Fig. 2. Correlation between the blood viscosity and the LDL-cholesterol plasma concentrations. The results show that milk decreases blood viscosity values but with the phytosterols ingestion the values are similar to those in the control group.

ingestion of milk supplied with phytosterols, comparatively to the placebo group, leads to a decrease in the HDL plasma levels. The plasmatic LDL-cholesterol levels have statistically significant changes: a 54.5% increase in the placebo group; a 73.7% decrease with a phytosterols concentration of 0.2 g phytosterols/dl ( $p < 0.0001$ ), 79.2% with 0.3 g phytosterols/dl ( $p < 0.0001$ ) and 74.8% with 0.4 g phytosterols/dl ( $p < 0.0001$ ) (Fig. 1).

### 3.3. Hemorheological parameters

Relatively to the hemorheological parameters the following results were obtained: an increase of the erythrocyte membrane fluidity,  $p < 0.0001$  for HC probe and a decrease of the erythrocyte deformability comparatively to the control group,  $p < 0.00001$  for the placebo group, and  $p < 0.04$  for group 1 at 12 and 60 Pa shear stresses.

The plasma viscosity results show significant increases with milk ingestion. Blood viscosity values when compared with the LDL-cholesterol plasma concentrations (Fig. 2), decrease for high levels of LDL-cholesterol.

## 4. Discussion and conclusions

The ingestion of low-fat milk supplied with phytosterols by Wistar rats and the further analysis of the hemorheologic and biochemical parameters results show interesting findings. One is that the phytosterols when incorporated in milk maintain their cholesterol-lowering properties, as we can see by the decreases observed in LDL levels after the milk 30-day feeding period, without alterations on food and milk consumption, body weight gain and biochemical parameters (Table 1).

Concerning the hemorheological parameters some significant differences were obtained compared with the control group: an increase in erythrocyte membrane fluidity, probably due to the decrease of cholesterol in the erythrocyte membranes; and in agreement with results obtained by Abugo et al. [9] a decrease in erythrocyte deformability (Fig. 3). Regarding the blood and plasma viscosity, the results

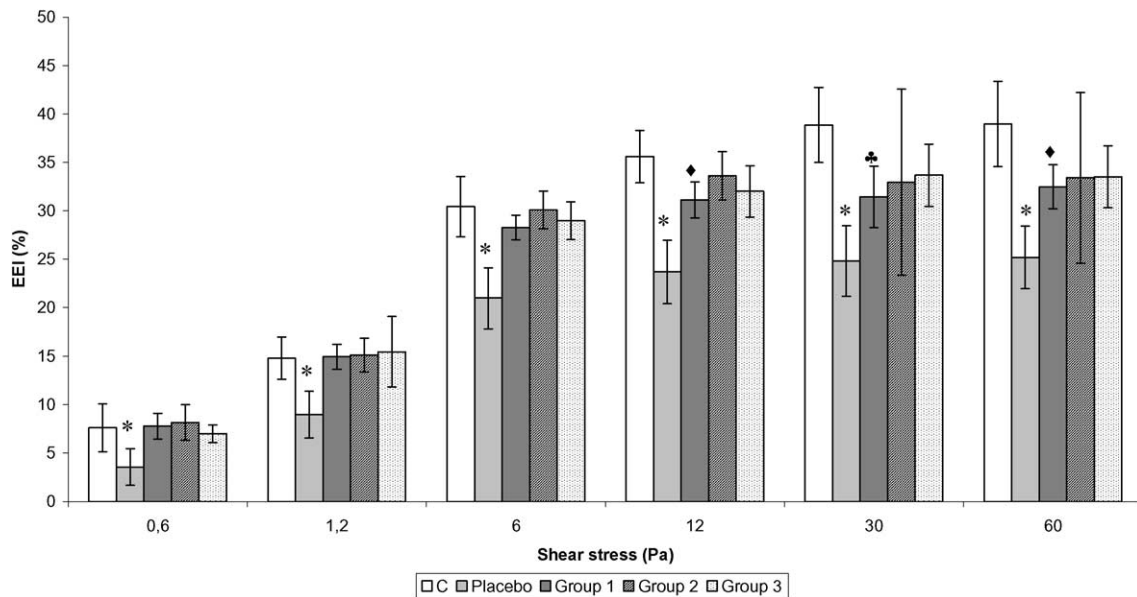


Fig. 3. Erythrocyte deformability measured by the erythrocyte elongation index (EEI). There are statistically significant changes in the placebo group (\* $p < 0.00001$ ) and in the group 1, (\* $p < 0.04$ ) for a shear stress of 12 and 60 Pa, and (\* $p < 0.009$ ) for a shear stress of 30 Pa. All the other values show a decrease, but not statistically significant.

Table 2

Values of erythrocyte membrane fluidity and plasma and blood viscosity (mean $\pm$ standard deviation) after a 30-day feeding period with low-fat milk

	Experimental groups				
	Control	Placebo	Group 1	Group 2	Group 3
DPH (sd)	0.309 $\pm$ 0.021	0.294 $\pm$ 0.019	0.314 $\pm$ 0.034	0.339 $\pm$ 0.034	0.296 $\pm$ 0.032
TMA-DPH (sd)	0.312 $\pm$ 0.018	0.326 $\pm$ 0.026	0.321 $\pm$ 0.017	0.315 $\pm$ 0.007	0.314 $\pm$ 0.009
HC (sd)	0.324 $\pm$ 0.061	0.259 $\pm$ 0.024	0.260 $\pm$ 0.041	0.186 $\pm$ 0.020	0.260 $\pm$ 0.045
		$p < 0.0008^\bullet$	$p < 0.0001^\bullet$	$p < 0.0002^\bullet$	$p < 0.0003^\bullet$
			NS $^\circ$	$p < 0.006^\circ$	NS $^\circ$
Plasma viscosity (mPa.s)*	1.09 $\pm$ 0.05	1.19 $\pm$ 0.12	1.16 $\pm$ 0.07	1.19 $\pm$ 0.07	1.15 $\pm$ 0.06
		NS $^\bullet$	$p < 0.004^\bullet$	$p < 0.0004^\bullet$	$p < 0.003^\bullet$
Blood viscosity 225 s $^{-1}$ (mPa)	5.20 $\pm$ 0.42	4.55 $\pm$ 0.42	5.36 $\pm$ 0.27	5.54 $\pm$ 1.10	4.97 $\pm$ 0.20
		$p < 0.005^\bullet$	NS $^\bullet$	NS $^\bullet$	NS $^\bullet$
			$p < 0.001^\circ$	$p < 0.02^\circ$	$p < 0.04^\circ$

$^\circ$  Statistically significant difference from the Control group;  $^\circ$  statistically significant difference from the Placebo group;

$^\bullet$  no statistically significant difference from the placebo group.

show a significant increase of plasma viscosity with milk ingestion and a decrease of blood viscosity for high levels of LDL (absence of phytosterols). With these results we may infer that milk should not be introduced in rats' diet due to its bad influence on lipid metabolism, namely the increase of the LDL-cholesterol plasma levels (Fig. 1). While the experimental animal model traditionally used for atherosclerosis studies has been the rabbit, with our study with rats we could obtain two different models, such as hypo- and hypercholesterolemia states. As can be seen in Fig. 2, for almost the same values of blood viscosity we have low LDL levels. With normal milk the LDL levels increase significantly and

blood viscosity decrease. The incorporation of phytosterols changes some of the alterations produced by milk ingestion, such as blood viscosity (Table 2) and erythrocyte deformability (Fig. 3). In conclusion the results show that low-fat milk containing phytosterols could be considered a hypolipemic food component, for further use in humans.

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### References

- [1] A.H. Sepowitz, S. Chien and F.R. Smith, Effects of lipoproteins on plasma viscosity, *Atherosclerosis* **38** (1981), 89–95.
- [2] C. Saldanha, J. Loureiro, C. Moreira and E.J. Martins, Silva, Behaviour of red blood cell aggregation in presence of lipoproteins, *Clin. Hemorheol.* **11** (1991), 543.
- [3] R.P. Mensink, S. Ebbing, M. Lindhout, J. Plat and M.M.A. van Heusten, Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations, *Atherosclerosis* **160** (2002), 205–213.
- [4] R. Volpe, L. Nittynen, R. Korpeia, et al., Effects of yoghurt enriched with plant sterols on serum lipids in patients with moderate hypercholesterolaemia, *Br. J. Nutr.* **86** (2001), 233–239.
- [5] P. Nestel, M. Cehun, S. Pomeroy et al., Cholesterol lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low fat foods, *Eur. J. Clin. Nutr.* **55** (2001), 1084–1090.
- [6] O.A. Matvienko, D.S. Lewis, M. Swanson, B. Arndt, D.L. Rainwater, J. Stewart and D.L. Alekel, A single daily dose of soybean phytosterols in ground beef decreases serum total cholesterol and LDL cholesterol in young, mildly hypercholesterolemic men, *Am. J. Clin. Nutr.* **76** (1) (2002), 57–64.
- [7] J. Harkness, A new instrument for measurement of plasma viscosity, *Lancet* **16** (1963), 280–281.
- [8] G. Schiliro, S. Livalti, E. Geremia, L. Masoti, A. Vanella and L. Mollica, Fluorescence studies on erythrocyte membranes from normal and thalassemic subjects, *IRCS Med. Sci.* **9** (1981), 599.
- [9] O.O. Abugo, R.R. Peddada, J.F. Kelly, G.S. Roth and J.M. Rifkind, Effect of cholesterol content in diet on capillary flow of rat erythrocytes, *Part I: Geometric and Flow Characteristics* **17** (1997), 437–443.

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