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The importance of the non-protein components of the diet in the plasma cholesterol response of rabbits to casein

Zinc and copper

BY S. SAMMAN AND D. C. K. ROBERTS

Human Nutrition Unit, Department of Biochemistry, University of Sydney, NSW 2006, Australia

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1. To characterize further the effect of protein–mineral interaction on plasma cholesterol, four groups of rabbits were placed on cholesterol-free, semi-purified diets for 12 weeks. The diets were similar in proximate composition with either casein or soya-bean protein isolate as the protein source (250 g/kg).

2. The dietary zinc content of the casein diets was increased to 22 mg/kg to equal that of the soya-bean diets. The copper content was set at 2 and 6 mg/kg. There were no differences in weight gain; all animals grew at a similar rate.

3. When the dietary Zn content was equalized, the plasma and liver cholesterol responses were similar regardless of the source of protein.

4. Differences in liver Cu and Zn were observed. As dietary Cu increased, Cu decreased and Zn increased in the liver. Hepatic Zn was also higher in soya-bean-fed than in casein-fed animals.

5. No effects on plasma Cu and Zn were seen.

6. The addition of Zn to the casein diet resulted in a similar plasma cholesterol to that of animals fed on the soya-bean diet, suggesting that casein-induced hypercholesterolaemia is due to a marginal Zn intake.

The effect of dietary protein on the plasma cholesterol of rabbits is well documented (Carroll *et al.* 1979; Kritchevsky & Czarnecki, 1982; Terpstra *et al.* 1983). Animal protein raises plasma cholesterol while protein from plant sources maintains it at a low level (Roberts, 1981; Terpstra *et al.* 1982). Several mechanisms have been suggested for this effect which include the amino acid composition of the protein source (Carroll & Woodward, 1982; Gibney, 1982; Sugano, 1983), and the reduced turnover of cholesterol (Huff & Carroll, 1980) and apolipoproteins (Roberts *et al.* 1981) in animals fed on casein as the source of protein. Differences in the digestibility and solubility of the proteins have also been implicated in the effect on plasma cholesterol of rabbits (West *et al.* 1984; Woodward & Carroll, 1985) and rats (Yashiro *et al.* 1985).

Recently, however, the potential role of minerals in this effect has been reported (Allotta *et al.* 1985; Van der Meer *et al.* 1985). Allotta *et al.* (1985) showed that rabbits fed on a casein diet with reduced salts develop a more pronounced hypercholesterolaemia than those fed on a standard casein diet. A protein–mineral interaction was also found, indicating a potential role for minerals in the casein-induced rise in plasma cholesterol. A similar interaction has been observed in the rat by Stemmer *et al.* (1985). They have shown that when diets containing minimal copper (0·5 mg/kg) with adequate zinc (20 mg/kg) were given to rats, the cholesterol response was greatest when casein was the protein source as compared with egg white or milk powder.

In contrast to the reduction in minerals causing a rise in plasma cholesterol (Allotta *et al.* 1985), Van der Meer *et al.* (1985) demonstrated that increasing the calcium content of a casein diet produced a reduction in plasma cholesterol. However, the involvement of other minerals cannot be excluded given the reported effects of Cu (for review, see Samman & Roberts, 1985), Zn (Philip *et al.* 1978; Koo & Williams, 1981) and other trace elements (Mertz, 1982) in cholesterol metabolism.

Thus as part of a continuing study investigating the effect of minerals on casein-induced hypercholesterolaemia, the Zn content of our casein diets was increased to equal that of the soya-bean-containing diets. The Cu content was set at two levels, low and adequate, 2 and 6 mg/kg respectively. Contrary to expectations, reducing the Cu content had no effect on plasma cholesterol. However, when the Zn content of the casein diet was increased, the expected rise in plasma cholesterol was negated. Thus these results are in agreement with our previous suggestion (Samman & Roberts, 1984) that supplementary Zn, not just Ca (Van der Meer, 1983), may make casein less hypercholesterolaemic. These findings, together with those reported by Van der Meer *et al.* (1985), also strengthen the notion that a protein–mineral interaction is responsible for casein-induced hypercholesterolaemia.

MATERIALS AND METHODS

Animals and diets

Young adult rabbits were obtained and housed under conditions previously reported (Allotta *et al.* 1985).

The Zn and Cu contents of the casein and soya-bean diets have been previously reported (Allotta *et al.* 1985), with the soya-bean diets having a higher amount of both these minerals. Because of this difference, the Zn content of the casein diets was increased to equal that of the soya-bean diets, that is 22 mg/kg. Although the Zn requirement of rabbits is not known ((US) National Research Council, 1977) and an amount of 68 mg/kg has been suggested for adequate growth during weaning (Hunt & Harrington, 1974), we have found that young adult rabbits grow adequately on as little as 12 mg/kg (Allotta *et al.* 1985). The Cu content was adjusted to 2 and 6 mg/kg.

The animals were randomly allocated to two groups of twelve animals and each group was then divided into two weight-paired subgroups of six animals, one group to be given the casein diet and the other the soya-bean counterpart. The animals were then weaned over 3 d on to the diets (the compositions of which have been reported by Allotta *et al.* 1985). They were maintained on these diets for 12 weeks and allowed 100 g/d and free access to deionized water. During the study two animals died due to respiratory infections unrelated to diet.

Cu and Zn analyses

Cu and Zn in the plasma, liver and diets were determined by atomic absorption spectrophotometry as previously reported (Allotta *et al.* 1985).

Lipid analysis

Representative samples of liver were extracted (Folch *et al.* 1957). Extracted cholesterol, following the evaporation of solvent and redissolving in propan-2-ol, was estimated using an enzymic method (Monotest cholesterol CHOD-PAP method, Boehringer Mannheim, W. Germany).

Statistics

Data were analysed by analysis of variance. Protein and Cu effects were tested as well as a protein–Cu interaction. An adjustment for unequal numbers was made by the method of Snedecor & Cochrane (1968).

Table 1. *Body-weight and the effect of diet composition on weight gain of rabbits after 12 weeks*

(Mean values with 95% confidence intervals)

			Initial wt (kg)		Final wt (kg)		Wt gain† (kg)	
Diet*	Copper (mg/kg)	*n*	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
Casein	2	5	1·95	0·07	2·54	0·25	0·58	0·28
Soya bean	2	6	1·87	0·14	2·44	0·24	0·57	0·15
Casein	6	6	2·00	0·13	2·34	0·14	0·38	0·18
Soya bean	6	5	2·40	0·29	2·81	0·19	0·44	0·30

* All diets contained 22 mg Zn/kg; for details of composition, see Allotta *et al.* (1985).
† No significant difference in weight gain (ANOVA).

Table 2. *Effect of equalizing the zinc content of the diet on the change in plasma cholesterol concentration (mg/l) of rabbits*

(Mean values with 95% confidence interval; results are expressed as absolute values for week 0 and changes from week 0 at week 6 and week 12)

Period on diet (weeks)...			0		6		12†	
Diet*	Copper (mg/kg)	*n*	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
Casein	2	5	890	240	430	1040	−130	700
Soya bean	2	6	720	480	−20	170	−330	520
Casein	6	6	630	370	630	420	420	820
Soya bean	6	5	310	190	−90	290	−150	170

* All diets contained 22 mg Zn/kg; for details of composition, see Allotta *et al.* (1985).
† No significant interactions after 12 weeks (ANOVA).

RESULTS

Weight gain and plasma cholesterol

Regardless of the type of dietary protein or the Cu content of the diets, all animals gained weight at a similar rate (Table 1). As in previously reported experiments, considerable individual variation in the change in plasma cholesterol concentration was seen in response to the various diets. With animals fed on either of the soya-bean diets and one (2 mg Cu/kg) of the casein diets there was a small mean decrease in plasma cholesterol after 12 weeks, while there was a similarly small increase in those fed on the remaining casein diet (6 mg Cu/kg) (Table 2). However, none of these differences reached statistical significance, indicating that when dietary Zn was increased in the casein diets to equal the Zn content of the soya-bean diet (Table 3), there were no observed differences in plasma cholesterol (Table 2) between any groups despite the difference in dietary Cu or protein.

Table 3. *Total copper and zinc contents of the diets (mg/kg) given to rabbits*
(Values are expressed as the mean of triplicate analyses)

Diet*	Cu Calculated	Cu Analysed	Zn Calculated	Zn Analysed
Casein	2	2·33	22	23·13
Soya bean	2	2·60	22	20·90
Casein	6	6·95	22	21·30
Soya bean	6	7·80	22	22·30

* For details of composition, see Allotta *et al.* (1985)

Table 4. *The effect of diet on liver wet weights (g) and liver cholesterol concentration of rabbits*

			Liver wet wt† (g)		Liver cholesterol† mg/g wet wt		Liver cholesterol† mg/total wt	
Diet*	Copper (mg/kg)	*n*	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
Casein	2	5	55·5	11·2	0·42	0·39	23	24
Soya bean	2	6	72·2	13·5	0·13	0·04	9	3
Casein	6	6	62·1	16·9	0·30	0·23	19	19
Soya bean	6	5	74·8	12·4	0·25	0·26	18	19

* All diets contained 22 mg Zn/kg; for details of composition, see Allotta *et al.* (1985).
† No significant difference (ANOVA).

Liver weight and cholesterol

Small variations in mean liver weight of the animals fed on the different diets were observed (Table 4) with those fed on casein being lower than those fed on soya bean. However, this difference did not reach statistical significance. In a similar manner to that seen for plasma cholesterol, liver cholesterol was not different between groups when expressed either as a concentration or as total amount (Table 4).

Liver Zn and Cu

The liver Zn (Table 5) concentration was affected by the dietary treatment with a significant Cu effect ($P < 0{\cdot}01$) and protein–Cu interaction ($P < 0{\cdot}01$). As dietary Cu increased, the rabbits fed on the diets containing 6 mg Cu/kg had a statistically higher mean concentration of Zn in the liver than those fed on the diets containing 2 mg/kg. This difference remained when total Zn in the liver was calculated.

Similarly differences were seen in the liver Cu concentrations, with a significant Cu effect ($P < 0{\cdot}01$). At a dietary Cu intake of 2 mg/kg, regardless of the source of protein, the mean liver Cu concentration and content were higher than in those rabbits fed on the diet with 6 mg Cu/kg. There was also a protein effect, with those fed on soya bean having a higher mean content of Cu in the liver than those fed on casein.

Table 5. *Effect of diet composition on the concentration of zinc and copper in the liver of rabbits*

(Mean values with 95% confidence intervals)

			Liver Zn				Liver Cu			
			μg/g wet wt		mg/total wt		μg/g wet wt		mg/total wt	
Diet†	Cu (mg/kg)	*n*	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
Casein	2	5	26·20	6·14	1·41	0·13	3·71	0·92	0·20	0·04
Soya bean	2	6	19·05	3·49	1·37	0·35	3·46	1·26	0·25	0·10
Casein	6	6	25·10	2·60	1·56	0·49	2·03	0·81	0·13	0·06
Soya bean	6	5	41·60	12·50	3·17	1·21	2·83	0·59	0·22	0·08
Terms										
Protein			NS		NS		NS		**	
Cu			**		**		**		NS	
Interaction			**		NS		NS		NS	

NS, not significant.
Significantly different (ANOVA): ** $P < 0.01$.
† All diets contained 22 mg Zn/kg; for details of composition, see Allotta *et al* (1985).

Table 6. *Effect of diet composition on plasma zinc and copper concentrations (μg/ml) of rabbits*

(Mean values with 95% confidence intervals)

			Plasma Cu		Plasma Zn	
Diet*	Cu (mg/kg)	*n*	Mean	95% confidence interval	Mean	95% confidence interval
Casein	2	5	0·50	0·26	0·72	0·17
Soya bean	2	5	0·46	0·09	0·86	0·18
Casein	6	6	0·75	0·37	0·84	0·14
Soya bean	6	5	0·58	0·14	0·94	0·13

* All diets contained 22 mg Zn/kg; for details of composition, see Allotta *et al.* (1985).

Plasma Cu and Zn

Unlike the liver, plasma Cu and Zn concentrations were similar for all dietary groups (Table 6). However, there was a trend for mean Cu to be higher in those fed on the diets containing 6 mg Cu/kg compared with those fed on 2 mg/kg.

DISCUSSION

The effect of dietary protein on plasma cholesterol in rabbits is well established, with casein inducing hypercholesterolaemia and soya bean maintaining a low concentration of plasma cholesterol (Carroll, 1982; West *et al.* 1982; Kritchevsky *et al.* 1983). However, in attempting to determine the mechanism, we found that the casein diet contains less Cu and Zn than the soya-bean counterpart (Allotta *et al.* 1985). When the Zn content was equalized, regardless of the Cu content, no significant differences in plasma or liver cholesterol were observed between casein-fed or soya-bean-fed animals. However, the absence of statistical significance may be due to small numbers in the groups. Nonetheless, this suggests that the reduction in dietary Cu from 6 to 2 mg/kg was not substantial enough to cause a Cu deficiency and the associated increase in plasma cholesterol (Nielsen *et al.* 1982; Lei, 1983). The increase in the Zn:Cu ratio from 3·5 to 11 did not affect plasma cholesterol in either casein-fed or soya-bean-fed animals. This is consistent with the view that the hypercholesterolaemic effect of the Zn:Cu ratio is only operative at a deficient level of Cu (Samman & Roberts, 1985). Thus, since additional Zn negated the hypercholesterolaemic effect of casein, we suggest that marginal Zn intake is responsible for casein-induced hypercholesterolaemia. However, a similar suggestion relating Ca to this effect has been reported (Van der Meer *et al.* 1985) and although the Ca content of our casein and soya-bean diets was not different (1·09 and 1·03 mg/kg respectively; mean of four samples), a Ca–Zn interaction cannot be ruled out (Samman & Roberts, 1984).

Manipulating the Cu and Zn contents of the diets resulted in variations in the liver concentrations of both elements. Increased dietary Cu resulted in a significant increase in the Zn content of the liver, an increase which was more pronounced in rabbits fed on soya bean than in those fed on casein. Thus there appears to be conservation of liver Zn at the higher intake of Cu, a similar effect to that shown in rats (Klevay *et al.* 1985). At a low Cu intake no differences were observed in liver Zn between casein-fed and soya-bean-fed animals. However, increased dietary Cu resulted in a decrease in liver Cu content and concentration with the decrease more pronounced in those fed on casein. A decrease in hepatic Cu concentration has also been shown in the rat when dietary Cu is increased from 1 to 2 mg/l, when Zn is 2·5 or 10·0 mg/l with egg white as the source of protein (Murthy *et al.* 1974).

Thus increasing dietary Cu from 2 to 6 mg/kg while maintaining the dietary Zn concentration resulted in an increase in Zn and a decrease in Cu concentrations in the liver. These hepatic changes were not reflected in the plasma nor did they have any apparent effect on plasma total cholesterol.

The use of diets equalized in Zn has strengthened the idea that a protein–mineral interaction may be involved in casein-induced hypercholesterolaemia in rabbits (Allotta *et al.* 1985). More specifically, we suggest that casein-induced hypercholesterolaemia is due to a marginal intake of Zn.

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