

# Erythrocyte Membrane Fluidity and Tissue Lipid Peroxides in Female Guinea-Pigs on Graded Vitamin C Intake

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*Received September 15, 1993*

*Accepted November 23, 1993*

## Summary

The effect of low (1 mg/animal/day), medium (10 mg/animal/day) and high (100 mg/animal/day) intake of ascorbic acid on tissue lipid peroxidation (LPO) and the physical state of erythrocyte membranes was investigated in female guinea-pigs fed a vitamin E low diet. Animals were killed after 9–11 weeks and the blood, liver, lungs, kidneys and adrenals were analysed. The LPO was estimated by the determination of malondialdehyde with HPLC. The physical state of erythrocyte membranes was determined spectrofluorometrically and expressed as fluorescence polarization of membrane lipid specific probe 1,6-diphenyl-1,3,5-hexatriene. The LPO concentrations in the liver and adrenals of the group on a low vitamin C intake were significantly increased. A significant non-linear negative correlation between C vitamin levels and LPO concentrations was found in these tissues. The fluidity of erythrocyte membranes as a measure of their structural state was significantly lower in the group with a low intake of C vitamin. It is probable that the water-soluble antioxidants, such as vitamin C, act in the plasma as primary defense against oxidative stress if the radicals are formed initially in the aqueous phase of whole blood.

## Key words

Membrane fluidity – Erythrocytes – Vitamin C – Guinea-Pig – Lipid peroxidation

## Introduction

Increased formation of oxygen free radicals and lipid peroxidation (LPO) accompanies tissue injury in most human diseases including atherosclerosis (Steinberg *et al.* 1989). Vitamin C has multiple antioxidant properties and significantly lowers the formation of LPO in male guinea-pigs exposed to carbon tetrachloride, polychlorinated biphenyls or cadmium (Kunert and Tappel 1983, Kato *et al.* 1981, Hudecová and Ginter 1992). On the other hand, ascorbic acid also shows prooxidant properties which involve vitamin C interaction with transition metal ions (Halliwell *et al.* 1992). Vitamin C can act as an antioxidant or as a prooxidant in microsomal lipid peroxidation and tocopherols may play a significant role in switching ascorbic acid from a potentially damaging agent to a protective one (Wefers and Sies 1988).

The aim of the present experiments was to investigate the response of tissue LPO and erythrocyte membranes rich in polyunsaturated fatty acids to low, medium and extremely high intake of vitamin C in guinea-pigs fed a diet low in vitamin E. Female guinea-

pigs, in which the effect of different doses of vitamin C on LPO has not yet been described, were used in these experiments. Sex differences in membrane susceptibility to lipid peroxidation have been reported in other animal species (Toyokuni *et al.* 1990, Hall *et al.* 1991).

## Materials and Methods

Tricoloured female guinea-pigs (Velaz, Prague) with initial body weight of about 400 g were housed under standard laboratory conditions in plastic cages with wood chip bedding. During the adaptation period, animals were fed *ad libitum* a standard laboratory chow for guinea-pigs (Mok, Velaz). Animals had free access to drinking water with 20 mg ascorbic acid/litre. Four weeks later, the animals with body weight of about 500 g were randomly divided into three subgroups and fed a vitamin C free diet *ad libitum* (Hudecová and Ginter 1992) with a low content of  $\alpha$ -tocopherol (1.4 mg/kg diet). The group 1 (low C) was given drinking water that provided ascorbic acid in

a mean dose of 1 mg per animal per day, group 2 (medium C) drinking water with 10 mg ascorbic acid per animal per day and group 3 (high C) drinking water with 100 mg ascorbic acid per animal per day. The intake of drinking water and vitamin C was controlled (Roe and Kuether 1943). Animals were weighed each week.

After 9, 10 and 11 weeks 4 animals from each subgroup were killed by decapitation after overnight fasting. The liver, lungs, kidneys and adrenals were excised, rinsed in ice-cold saline solution and immediately prepared for estimation of LPO by determining malondialdehyde (MDA), which is a breakdown product of LPO. MDA was determined after the reaction with thiobarbituric acid using HPLC (Hewlett-Packard HP 1090) separation of the reaction product (Ohkawa *et al.* 1979, Wong *et al.* 1987). The concentration of vitamin C (ascorbic + dehydroascorbic acid) was determined in the same tissues (Roe and Kuether 1943). Altogether, 12 animals were analysed in each group.

#### Preparation of erythrocyte membranes

Blood was collected directly into heparinized tubes and immediately washed three times with Tris buffer (pH 7.6, 310 mOsm). The washed erythrocyte suspension (5 ml, haematocrit 50 %) was haemolysed with 30 ml of ice-cold Tris buffer (pH 7.6, 20 mOsm) and centrifuged at 20 000 x g for 40 min at 4 °C. Washing was repeated four times to obtain a colourless membrane preparation (Hanahan and Ekholm 1974). Membrane preparations of 4 individual animals were pooled for fluorescence polarization measurements.

#### Incorporation of the fluorescent probe into membranes

1,6-diphenyl-1,3,5-hexatriene (DPH, Serva), was dissolved in tetrahydrofuran of spectral grade to obtain

2 mmol/l solution. The stock solution was protected from light and stored in the cold. 0.1 ml of this solution was injected into 100 ml of 0.15 M NaCl with intensive stirring for 15 min and immediately used. 2 ml of probe solution were added into 2 ml of cell suspensions. The final the probe concentration in a sample was  $1.10^{-6}$  mol/l. The mixture was then incubated for 1 h in the dark at room temperature.

#### Fluorescence measurements

Fluorescence intensities and polarization values were measured on a Perkin Elmer LS-5 spectrofluorometer equipped with polarization accessory. The excitation monochromator was set at 355 nm and the emission was recorded at 445 nm. The slits of both monochromators were 5 nm wide. The samples were measured in 10x10 mm quartz cells at room temperature.

The degree of fluorescence polarization (P) was calculated according to Shinitzky (Shinitzky and Barenholz 1974, Shinitzky and Inbar 1974) using the equation:  $P = (I_0 - I_{90}G)/(I_0 + I_{90}G)$  where  $I_0$  and  $I_{90}$  are the intensities of the emitted light the polarization plane of which is oriented parallel and perpendicular to the polarization plane of the excitation beam, respectively. G is the correction (grating) factor for instrument light scattering. A high degree of fluorescence polarization (P) represents a high structural order indicating low membrane fluidity and *vice versa* (Antunes-Madeira *et al.* 1990). Measurements were always corrected for the contribution of sample light scattering using samples with suspended cells but without added probe.

The results were evaluated statistically by the analysis of variance and regression analysis. The level of significance was set at  $P < 0.05$ .

**Table 1**

Vitamin C and LPO levels in the organs of female guinea-pigs on graded ascorbic acid intake

Parameter	Group	Liver	Lungs	Kidney	Adrenals
Vitamin C (nmol/g wet tissue)	1: Low C	74 ± 17 <sup>a</sup>	250 ± 45 <sup>a</sup>	85 ± 17 <sup>a</sup>	545 ± 102 <sup>a</sup>
	2: Medium C	295 ± 34 <sup>b</sup>	682 ± 45 <sup>b</sup>	205 ± 17 <sup>b</sup>	1989 ± 267 <sup>b</sup>
	3: High C	989 ± 68 <sup>c</sup>	1676 ± 80 <sup>c</sup>	619 ± 34 <sup>c</sup>	5403 ± 392 <sup>c</sup>
Lipid peroxidation (nmol/g wet tissue)	1: Low C	29 ± 3 <sup>a</sup>	14 ± 1 <sup>a</sup>	28 ± 3 <sup>a</sup>	81 ± 7 <sup>a</sup>
	2: Medium C	20 ± 1 <sup>b</sup>	12 ± 1 <sup>a</sup>	26 ± 1 <sup>a</sup>	56 ± 6 <sup>b</sup>
	3: High C	20 ± 2 <sup>b</sup>	12 ± 1 <sup>a</sup>	24 ± 2 <sup>a</sup>	43 ± 6 <sup>b</sup>

Group 1: Low C – 1 mg ascorbic acid/animal/day, Group 2: Medium C – 10 mg ascorbic acid/animal/day, Group 3: High C – 100 mg ascorbic acid/animal/day, <sup>a,b,c</sup>: Different indices indicate significantly different means ( $P < 0.05$ ) in the same column. Concentrations (mean ± standard error) are presented for 12 animals per group.

Results and Discussion

Body weights, which increased during the experiment by 100–150 g in all three groups did not differ among the groups. The appearance of the experimental animals in all three groups was without observable changes during the whole experiment. After 9 weeks a steady state was probably achieved because the concentrations of vitamin C and LPO were practically the same in the subgroups sacrificed after 9, 10 or 11 weeks of the experiment. The results obtained in these time intervals were thus pooled (Table 1).

Graded intake of ascorbic acid led to graded vitamin C concentrations in all tissues. Vitamin C levels in group 1 (low C) were higher than in guinea-pigs with acute scurvy but were very similar to those in guinea-pigs with marginal vitamin C deficiency (Ginter *et al.* 1979). In group 2 (medium C), with a 10 times higher ascorbic acid intake, the tissue levels of vitamin C were 2–4 times higher in comparison to group 1. In group 3 (high C) with 100 times higher ascorbic acid intake, vitamin C levels were 6–12 times higher in comparison to group 1, but they did not reach saturation levels (Ginter *et al.* 1979, Berger *et al.* 1989).

and adrenals in group 1 is noteworthy because the experimental animals were healthy and unexposed to any toxic material. Moreover, they were females which are more resistant to oxidative stress (Toyokuni *et al.* 1990, Hall *et al.* 1991). It is probable that suboptimal intake of ascorbic acid caused the accumulation of LPO because of the suboptimal concentration of vitamin E in the diet (McCay 1985).

The accumulation of LPO in the critical organs led us to the assumption that the cell membrane could also be influenced by lipid peroxidation. As a model, the erythrocyte membrane was chosen. Changes in membrane fluidity could reflect changes in the lipid moiety of the membrane. The erythrocytes are susceptible to oxidation. Their membranes are rich in polyunsaturated fatty acids and are continuously exposed to high oxygen concentrations. It was found that the erythrocyte membranes were oxidized by a free radical chain mechanism (Yamamoto *et al.* 1985). These oxidations affect the chemical and physical properties of the membrane. The *cis,cis*-polyunsaturated fatty acids are converted to *cis,trans*-, or *trans,trans*-isomers (Porter 1986). The phospholipid liposomal membranes become more rigid and less fluid as the oxidation proceeds (Takahashi *et al.* 1988).

The fluorescence polarization of lipid specific probe DPH values are listed in Table 2. There is an evident decrease of polarization values which represent an increase of membrane fluidity with increasing vitamin C levels. The fluidity of erythrocyte membranes was significantly lowered in the group with a suboptimal intake of vitamin C as compared to the groups with a higher vitamin C intake. These results confirm the presumption that the water-soluble antioxidants in the plasma, such as vitamin C, act as primary defense against the oxidative stress when the radicals are formed initially in the aqueous phase of the whole blood.

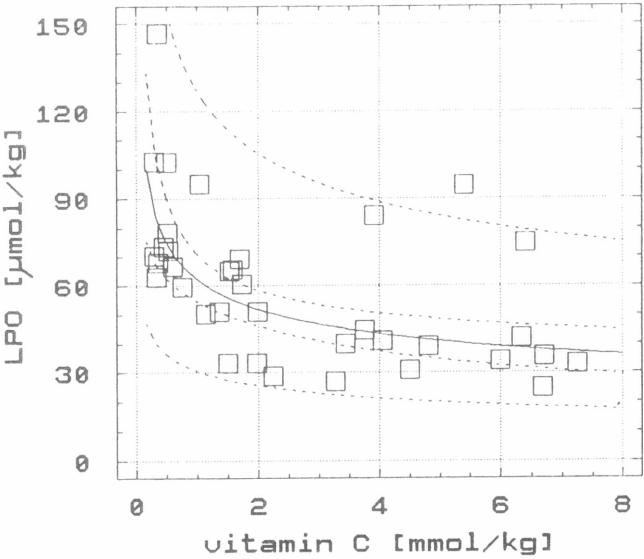


Fig. 1  
Correlation between vitamin C and LPO levels in adrenals (multiplicative model,  $r=0.632$ ,  $p<0.0001$ ). Confidence and prediction limits appear on the regression plot as the pair of dashed lines closest to and farthest from the regression curve, respectively.

LPO concentrations in the liver and adrenals of group 1 (low C) were significantly increased in comparison to groups 2 and 3. In these organs a significant non-linear negative correlation between vitamin C levels and LPO concentrations was found (Fig. 1). The finding of the high LPO level in the liver

Table 2  
Fluidity of erythrocyte membranes of guinea-pigs on graded vitamin C intake expressed as polarization of lipid specific fluorescence probe DPH.

Intake of AA	Polarization P	S.E.M.
1 mg/day	0.377 <sup>a</sup>	0.006
10 mg/day	0.353 <sup>b</sup>	0.005
100 mg/day	0.351 <sup>b</sup>	0.005

AA Ascorbic acid, S.E.M. = standard error of the mean, <sup>a,b</sup>: Different indices indicate significantly different means ( $p<0.05$ ) in the same column

In the guinea-pigs unexposed to toxic material, the daily intake of 10 mg ascorbic acid per animal, i.e.

approximately 20 mg/kg body weight (data from group 2, Tables 1 and 2), is probably sufficient for antioxidant defense. The optimum requirement of ascorbic acid for the prevention of lipid peroxidation and other toxic effects could be several times higher in animals exposed to polychlorinated biphenyls, cadmium or other toxic substances (Kato *et al.* 1981, Hudecová and Ginter 1992). Our results do not confirm the apprehension of some authors from the prooxidant effects of high doses of vitamin C (Baysal *et al.* 1989). In our group 3 (high C), fed with 100 mg of ascorbic acid/animal/day, i.e. about 200 mg/kg of body weight, neither the increase of LPO levels nor the unfavourable changes in erythrocyte membranes, were observed despite the suboptimal concentration of vitamin E in the used diet.

The prooxidant and mutagenic activity of ascorbic acid has often been established using simple *in vitro* systems containing transition metal ions (copper or iron) (Kunimoto *et al.* 1981). *In vivo*, the

antioxidant properties of ascorbic acid normally predominate, but this need not be true in pathological states where transition metals become more available (Halliwell *et al.* 1992). The different turnover rate of ascorbate in guinea-pigs and man does not make it possible to extrapolate these guinea-pig data to man directly. However, it is probable that the marginal vitamin C deficiency increases the risk of LPO production even in man. For the prevention of lipid peroxidation in people exposed to toxic substances and in smokers, the daily intake of vitamin C should be higher than the officially recommended values (Harats *et al.* 1990, Ginter 1989).

### Acknowledgement

The authors thank to Ing. A. Hudecová and L. Hudecová for LPO analysis and Mrs. B. Hatalová for statistical evaluation of the results and for the preparation of the manuscript.

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