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Title Page

Ascorbylperoxide contaminating parenteral nutrition perturbs the lipid metabolism in newborn guinea pig

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Running Title Page

Running title: **Ascorbylperoxide on glycolysis and lipogenesis**

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Abbreviations: ACC: acetyl-CoA carboxylase; Ascorbylperoxide: 2,3-diketo-4-hydoxyperoxyl-5,6-dihydroxyhexanoic acid (structure in Knafo et al., 2005); GK: glucokinase; GSH: reduced form of glutathione; GSSG: disulfide form of glutathione; PFK: phosphofrutokinase; TG: triacylglyceride.

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Abstract

The light exposure of parenteral nutritive solutions generates peroxides such as H_2O_2 and ascorbylperoxide. This absence of photo-protection is associated with higher plasma triacylglycerol concentration (TG) in premature infants and, in animals, with oxidative stress and a H_2O_2 independent hepatic steatosis. We hypothesized that ascorbylperoxide is the active agent leading to high TG. The aim was to investigate the role of ascorbylperoxide on glucose and lipid metabolism in an animal model of neonatal parenteral nutrition. Three day-old guinea pigs received, through a catheter in jugular, solutions containing dextrose+ 0, 90, 225 or 450 μM ascorbylperoxide. After 4 days, blood and liver were sampled and treated for determinations of TG, cholesterol, markers of oxidative stress (redox potential of glutathione & $F_{2\alpha}$-isoprostane) and activities & protein levels of acetylCoA carboxylase (ACC), glucokinase and phosphofructokinase (PFK). Ascorbylperoxide concentration was measured in urine of the last day. Data were compared by ANOVA, $p<0.05$. Plasma TG and cholesterol as well as hepatic PFK activity increased (200% of control), whereas ACC activity decreased (66% of control), in function of amount of ascorbylperoxide infused. Both markers of oxidative stress were higher in animals receiving the highest ascorbylperoxide. The logarithmic relations between urinary ascorbylperoxide and plasma TG ($r^2=0.69$) and hepatic PFK activity ($r^2=0.26$) were positive, whereas it was negative with ACC activity ($r^2=0.50$). In conclusion, the ascorbylperoxide contaminating the parenteral nutrition stimulates glycolysis allowing higher availability of substrates for lipid synthesis. The logarithmic relation between urinary ascorbylperoxide and plasma TG suggests a very low efficient concentration.

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Introduction

Intravenous nutritive support is essential for those who cannot be fed by mouth. This mode of nutrition is frequent in premature infants born before 28 weeks of gestation in whom the gastro-intestinal track is immature. Parenteral nutritive solutions are compounded to provide all essential nutrients to sustain the growing and development of neonates (Gunn et al., 1978). However, as administered, without adequate photo protection, some nutritive elements are lost and undesirable new molecules are generated. Indeed, exposure to ambient light induces a loss of antioxidant vitamins (Silvers et al., 2001) and generates nutrients oxidation by-products such as peroxides (Helbock et al., 1993; Lavoie et al., 1997-2004**,** Neuzil et al., 1995; Knafo et al., 2005). Paradoxically, the main source of peroxides is the multivitamins moiety of parenteral nutritive solution (Lavoie et al., 1997). The photo-excited riboflavin catalyzes the electron transfer to dissolved oxygen to produce superoxide anion and singlet oxygen which react with electron donors such as lipid, amino acids and ascorbate to generate peroxide (Helbock et al., 1993; Knafo et al., 2005; Laborie et al., 1998; Lavoie et al., 1997). Eighty percent of these peroxides are H_2O_2 (Lavoie et al., 1997).

In infants, the absence of photo-protection of parenteral nutrition is associated with higher concentration of peroxide in urine (Laborie et al, 2000), glucose in blood and triacylglycerol in plasma (Khashu et al, 2009). These observations were prevented by a light protection from the preparation of the nutritive solution to the delivery to the infants. In animals, photo-exposed parenteral nutrition induced oxidative stress (Lavoie et al., 2000; Chessex et al., 2001) and hepatic steatosis (Chessex et al., 2002). Since this fatty liver was not associated to H_2O_2 contaminating the infused solutions (Chessex et al., 2002), a new compound deriving from the action of light on parenteral nutrition was suspected. Knafo et al (2005) reported that the interaction between dehydroascorbate and H_2O_2, both generated during light-exposure,

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leads to the formation of the new molecule 2,3-diketo-4-hydoxyperoxyl-5,6-dihydroxyhexanoic acid, named ascorbylperoxide. The concentration of this molecule rises in urine of animal infused with increasing amount of parenteral multivitamins and was associated with the activation of the acetylCoA carboxylase (ACC), a limiting enzyme of lipogenesis.

We hypothesized that the high plasma triacylglycerol concentration (TG) observed in premature infants receiving a parenteral nutritive solution without photo-protection is caused by ascorbylperoxide contaminating their parenteral nutrition. The implication of oxidative stress is suspected since ascorbylperoxide has an oxidative (peroxide) function. The present study wanted to investigate the lipogenic effect of ascorbylperoxide in animal model of neonatal parenteral nutrition by infusing during four days a solution containing dextrose + ascorbylperoxide. Since, here, the only carbon source for TG synthesis was glucose, the aim of the study was to measure TG in function of oxidative stress markers as well as the activity of key enzymes of lipogenesis (ACC) and glycolysis (glucokinase and phosphofructokinase).

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Methods

Animal Model and protocol

Three-day old Hartley guinea pigs (Charles River, St-Constant, Qc, Canada) received through a catheter in jugular, a continuous intravenous infusion (220 mL/kg/d) of basal solution (8.7% (w/v) dextrose + 0.35% (w/v) NaCl + 1U/mL heparin) enriched with ascorbylperoxide (0, 90, 225 or 450 µM); they were not enterally fed. Animals were on 12:12 hours light: dark cycle and the solutions were changed daily four hours after beginning the light cycle. This model was previously used to study the impact of parenteral nutrition or peroxides on hepatic oxidative stress (Chessex et al, 2001) and lipid metabolism (Chessex et al, 2002). Urine of the last day was collected for ascorbylperoxide determination. After four days, under anesthesia (ketamine/xylazine), blood was sampled from aortic puncture whereas the liver was washed from blood by flushing 30 mL of cold 0.9% NaCl solution from hepatic vein to portal artery, collected and rapidly minced on ice. One aliquot was treated for glutathione determination whereas the remaining hepatic tissues were separated in different fractions. Liver samples as well as plasma aliquots were kept at -80ºC until biochemical determinations. The protocols were carried out in accordance with the Canadian Council of Animal Care guidelines.

Ascorbylperoxide generating system

Ascorbylperoxyde (2,3-diketo-4-hydoxyperoxyl-5,6-dihydroxyhexanoic acid, structure in Knafo et al., 2005) is a new molecule identified in solutions containing ascorbate such as parenteral nutrition (: Lavoie et al., 2004; Knafo et al., 2005). The pure molecule does not commercially exist. Therefore, for the purpose of the study the following generating system was used. According to characteristics of the chemical reaction (Knafo et al., 2005), a solution containing 1.8 mM ascorbate + 400 µM H_2O_2 + 30 µM riboflavin (pH 7 adjusted with NaOH 1N) was incubated at room temperature with strong stirring (to favor dissolution of

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atmospheric oxygen) under ambient light exposure (75 foot-candle) for 48 hours (for complete consumption of ascorbate confirmed by mass spectrometry). A similar system, but without ascorbate, was used to generate the control solution (no ascorbylperoxide). The solutions were treated 20 min with 100 U/mL catalase and filtered against a 30 kDa filter Centricon Plus-20 (Millipore Corporation); the absence of H_2O_2 was controlled using the FOX assay (Jiang et al, 1991). A dilution of this solution generating 1800 µM ascorbylperoxyde was used to prepare the final solutions containing 90, 225 or 450 µM ascorbylperoxide. The infused solutions were replaced each day by new freshly prepared solutions.

Analytical Measurements

Ascorbylperoxide

Based on previous study (Knafo et al., 2005), ascorbylperoxide in infused solution as well as in urine was quantified by using Agilent LC/MS 1100 mass spectrometer and L-2-oxo-thiazolidine 4-carboxilic acid (OTC) (Aldrich Chemical), as internal standard. Urine samples were centrifuged 1 min at 7000 x G before used. Five µL of 2.5 mM OTC were added to 95 µL of sample and 4 µL of this solution were injected on LC column ZORBAX Eclipse XDB C_{18} (Agilent). The elution was performed with isocratic mixture of ammonium acetate 10 mM (pH 7) : acetonitril (1:1) at 0.4 mL/min. Retention time was 2.6-2.8 min for ascorbylperoxide and 4.0 – 4.3 min for OTC. Ascorbylperoxide and OTC were quantified by monitoring ion abundance at m/z 207 (ascorbylperoxide), 147 (OTC) and 293 (OTC dimer) from electrospray ionization mass spectrometry (Agilent 1100 single quadrupole) (negative mode, 25V, source temperature of 350°C, nitrogen nebulizer gas flow of 12 L/min). The inter-assay coefficient of variation for ascorbylperoxide in urine was 4% whereas it was 11% for the intra-assay.

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Total OTC abundance (abundance at 147 m/z + 2 fold abundance at 293) was used as internal standard. Its linearity was excellent (r^2 >0.99) until 1 mM. The linear correlations between the ratio ascorbylperoxide / OTC (abundance / abundance) and the increasing amounts of OTC (ascorbylperoxide constant) or of ascorbylperoxide (OTC constant) were greater than 0.99. These linear correlations demonstrate the absence of interaction between OTC and ascorbylperoxide into the assay. The stability of OTC was not affected by the pH (2 to 9) or freezing at -20°C (until 168 days). Whereas ascorbylperoxyde concentration at -80°C decreases in function of time ($y=0.30\ \%\bullet d^{-1}\bullet x + 100\%$, $r^2 = 0.83$).

Glutathione

Immediately after sampling, 0.5 g of liver was homogenized (2 x 10 sec with Polytron Teador™) in 2.5 ml of iced and freshly prepared 5% (w/v) metaphosphoric acid and centrifuged 3 min at 7200 x g. Pellets and supernatants were kept at -80°C until protein and glutathione determinations respectively. GSH and GSSG were quantified by capillary (75µm x 50cm silica) electrophoresis (75 mM boric acid and 25 mM Bis-Tris, pH 8.4, 28°C, 18 kV) as previously described (Lavoie et al, 2008, Turcot et al, 2009). The redox potential refers here to the half-cell reduction potential of the GSSG (2H+/2GSH couple) and it was calculated by using the Nernst equation (25°C, pH 7.0) (Schafer et al, 2001).

8-Isoprostane $F_{2\alpha}$, triacylglycerol, total cholesterol, blood glucose

Isoprostane levels were determined by using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) as previously reported (Chessex et al, 2002), triacylglycerol and total cholesterol were measured by using an enzymatic colorimetric test from Roche Diagnostics (Turcot et al, 2009). Blood glucose was quantified with the OneTouch Ultra glucometer (Johnson & Johnson).

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AcetylCoA carboxylase activity

ACC activity was measured on the 6% PEG 8000 (Sigma-Aldrich) fraction (Kudo et al, 1995) as previously reported (Turcot et al, 2009). The measurement was based on the addition of carbon atom from ^{14}C bicarbonate ($NaH^{14}CO_3$; 14 mCi/mmol; Sigma-Aldrich) on acetyl-CoA and was expressed as nmol malonyl-CoA produced/min/mg protein. The assay was performed after pre-incubation in presence or not of 7.5 mM glutamate, which revealed the total activity of the enzyme by stimulating phosphatase protein 2A (Gaussin et al, 1996).

Glucokinase activity

The activity was determined according to the method described by Ferre et al (1996) and Xu Ming-Zhi et al (2004). Briefly, GK were measured spectrophotometrically on 23 000x g supernatant of liver homogenate in a system coupled with G-6-P dehydrogenase after a 20 min incubation at 37°C. Difference in activities obtained by using 100 and 0.5 mM glucose as substrate, corresponds to the glucokinase activity

Phosphofructokinase-1 activity

The activity was determined according to the method of Hamer et al (1987) and Karadesh et al (1977). Briefly, PFK-1 was assayed by the measurement of NADH oxidation at 340nm on 100 000 x g supernatant of liver homogenate in a system coupled with aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase.

Western blots – Phosphofructokinase and Glucokinase

According to the method of Abnous (2008) and Rideau (2008), 300 mg of liver were homogenized in 0.6 mL buffer (pH 7.4) containing 10 mM Tris, 1 mM EDTA, 1 mM EGTA,

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150 mM NaCl, 100 mM NaF, 2mM orthovanadate sodium, 1% (v/v) Triton X-100, 0.01% (v/v) SDS 10%, 2mM PSMF and protease inhibitor cocktail (1 complete tablet / 20ml; Roche Diagnostics). After one hour incubation at 4°C, the suspension was centrifuged (4°C) 30 min at 6300 x G. The supernatant was centrifuged (4°C) 45 min at 18 000 x g. Fifty µg from supernatant were separated on 8% polyacrylamide electrophoresis (100V - 90 min) and electroblotted onto PVDF membrane (90V - 2 hours at 4°C). The membrane was incubated overnight at 4°C with the first anti-body (polyclonal anti-human GK (Abnova Corporation, Taipei, Taiwan) (1: 1000), PFK-1(H-55) polyclonal (Santa-Cruz Biotechnology, CA, USA) (1:1000) and monoclonal anti-mouse vinculin (Abnova Corporation, Taipei, Taiwan) (1:2500)) and 1 hr with the second antibody (goat anti-mouse IgG-HRP secondary antibody (R&D Systems, Cedarlane Labs) (1:5000)). After washing (4x15 min), the second anti-body was revealed by chemiluminescence (25% SuperSignal West Femto : 75% SuperSignal West Pico; Pierce Biotechnology, Rockford, IL, USA) on X-ray film. The quantification was done by densitometry (Fluor Chem 8800 imaging system).

Western blot – AcetylCoA carboxylase

According to the method of Ouadda (2009), 50 mg of frozen liver were homogenized in 0.5 mL buffer (pH 7.5) containing, 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton-100 X, 50 mM NaF, 10% glycerol, 1mM PMSF, 5 µg/ml aprotenin, 5 µg/ml leupeptin and 1mM Na_3VO_4. After centrifugation (13 000 rpm, 20 min, 4°C), 25 µg of protein from supernatant were separated on 8% polyacrylamide electrophoresis (170V - 50 min), electro-blotted onto PVDF membrane (100V – 70 min at 4°C) and blocked (1 hour at room temperature) with skimmed milk at 5% in PBS-Tween20. The membranes were incubated overnight at 4°C with the polyclonal anti-human ACC (Cell Signaling Technology, MA, USA) (1: 500) and the monoclonal anti-mouse vinculin (Abnova Corporation, Taipei,

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Taiwan) (1:2500) followed by 1 hr incubation with goat anti-rabbit IgG-HRP secondary antibody (Promega Corporation, WI, USA) (1:20 000). After four washing (15 min), the result was revealed by chemiluminescence (100 % Western Lightning™ Chemiluminescence Reagent, PerkinElmer, MA, USA) on X-ray film. The quantification was done by densitometry (Fluor Chem 8800 imaging system).

Statistical analysis

Data were presented as mean ± s.e.m. and were compared by ANOVA after validation of homoscedasticity using Bartlett's Chi square test. Logarithmic transformation of GSH, GSSG and ACC Western blot data was used to meet homoscedasticity. The orthogonal comparisons used were [{(0 vs. 1 μmol/d ascorbylperoxyde group) vs. 4 μmol/d } vs. 7 μmol/d] in order to statistically document the lowest efficient concentration of ascorbylperoxide. The level of significance was set at $p < 0.05$.

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Results

The initial body weight (106 ± 3 g), as well as final body weight (100 ± 2 g) and liver weight (3.6 ± 0.1 g or 37.0 ± 1.8 mg/g of body weight), did not differ between groups ($F_{(1,23)}$<3.9). Blood glucose was similar ($F_{(1,19)}$<2.3) between groups (8.0 ± 0.4 mM).

The exact amounts of ascorbylperoxide received by animals were reported in **Table 1** and they are used in figures to qualify the four groups. Data shown that ascorbylperoxide is eliminated in urine. The concentration measured in urine from animals that did not be infused with ascorbylperoxide suggests an endogenous generation of this molecule. The threshold of this endogenous value (95% confidence) was 0.80 µmol/ mg creatinine or 54 µM. By deducting this limit to urinary concentration of ascorbylperoxide, the calculated recovery in urine increased in function of infused amount and reached a plateau greater than 80% with the infusion of 4 µmol/d (Table 1).

To challenge the hypothesis of an impact of ascorbylperoxide on TG, the experimental design allowed to investigate two options: modification of fatty acids synthesis, characterized here by the ACC activity, and modification of the substrate availability from glycolysis, characterized by GK and PFK activities, to sustain the lipid synthesis. The determination of TG and total cholesterol allowed distinguishing between the two options. Indeed, a modification in TG alone would suggest an effect of ascorbylperoxide on fatty acid synthesis whereas a similar modification of both lipids would suggest an impact on a common substrate. **Figure 1** shows that both TG (Figure 1A) and total cholesterol (Figure 1C) were increased in plasma of animals infused with ascorbylperoxide. The difference was significant with the infusion of 4 µmol/d for plasma TG ($F_{(1,17)}$=9.1) and of 1 µmol/d for plasma total cholesterol ($F_{(1,17)}$=9.6).

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No effect of ascorbylperoxide was observed in liver neither for TG (($F_{(1,17)}$<1.9) (Figure 1B) nor for total cholesterol ($F_{(1,17)}$<0.2) (Figure 1D).

ACC exists in its active and inactive (phosphorylated) form. The basal activity (non phosphorylated form) of ACC (**Figure 2A**) was negatively affected by infused ascorbylperoxide but only at the highest intake ($F_{(1,18)}$=12.3). The lost of activity can be explained by a more phosphorylated state or a lost of enzyme. The first possibility was investigated by measuring the activity of the enzyme in presence of glutamate to provide the total activity. The ratio of basal on total activity indicates the level of activation of the enzyme. The total activity (Figure 2B) was lower in animals infused with at least 4 µmol/d of ascorbylperoxide ($F_{(1,18)}$=7.4) whereas the ratio of the activities (Figure 2C) was already affected with the lowest amount of ascorbylperoxide infused ($F_{(1,18)}$=9.2), suggesting that ascorbylperoxide induces a de-phosphorylation of the enzyme. The activation of ACC by ascorbylperoxide has been already reported in our animal model (Knafo et al., 2005). On the other hand, the western blot analysis (Figure 2D) shows that the protein level of ACC was lower in animals infused with ascorbylperoxide ($F_{(1,18)}$=6.2). Together, these data suggest that ascorbylperoxide has induced a more active (less phosphorylated) ACC but in a lower protein amount, whose resultant was a weaker activity.

The second option that can explain increased lipid production was a greater availability of substrate. AcetylCoA, which can derive from glycolysis, is a common substrate for the synthesis of TG and cholesterol. Glycokinase (GK) is the key enzyme in liver to favor the glucose uptake by hepatocytes (Agius, 2008) whereas phosphofructokinase-1 (PFK) is the enzyme limiting the entry of glucose in glycolysis leading to formation of acetylCoA. The GK activity (**Figure 3A**) decreased ($F_{(1,19)}$=4.7) in animals infused with the highest amount of

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ascorbylperoxide (7 µmol/d), whereas 4 µmol/d were sufficient to induce a rise in PFK activity ($F_{(1,19)}$=9.1) (Figure 3B). The protein amount of both enzymes did not vary between groups ($F_{(1,19)}$<0.6) (Figures 3C & D). Plasma TG was not explained by PFK activity (linear correlation r^2 = 0.13) whereas it correlated significantly and negatively with ACC activity (y= -7.6 nmol•min^{-1}•mg prot^{-1}•mM^{-1}•x + 6.8 mM, r^2 = 0.37, p<0.01) (**Figure 4**).

The hepatic oxidative effect of ascorbylperoxide (**Figure 5**) has revealed with the highest amount infused (7 µmol/d). Indeed, in this group, isoprostane level (Figure 5B) as well as and the redox potential of glutathione (Figure 5A) was higher ($F_{(1,19)}$>6.7). The more oxidized status of glutathione in this group was associated with a lower tissue concentration of GSH ($F_{(1,19)}$=5.6) (**Table 2**) rather than a modification in GSSG level ($F_{(1,19)}$<3.8).

Figure 6 shows TG values (**A**) as well as the activities of PFK (**B**) and ACC (**C**) in function of urinary concentration of ascorbylperoxide. TG value and PFK activity increased positively in a logarithmic function with ascorbylperoxide (TG: y= 0.063ln(x)+0.20 mM, r^2 = 0.69, p<0.01; PFK: y= 1.1ln(x)+5.7 nmol•min^{-1}•mg prot^{-1}, r^2 = 0.26, p<0.05). In contrast, the logarithmic relation between ACC activity and urinary ascorbylperoxide was negative (y= -0.7ln(x)+5.4 nmol•min^{-1}•mg prot^{-1}, r^2 = 0.50, p<0.01).

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Discussion

An important finding of the study is that infusion of increasing amount of ascorbylperoxide is associated with a rise of plasma triacylglycerol and cholesterol concentrations as well as a stimulation of PFK activity. In contrast to what was expected, these effects does not seem to be occurred through an oxidative stress caused by this peroxide. Indeed, in concentration range affecting TG and PFK, two markers of oxidative stress that are isoprostane and redox potential of glutathione were affected only by the infusion with the highest concentration of ascorbylperoxide. More surprising was the drop of ACC activity in function of infused ascorbylperoxide. This last effect supports the notion that ascorbylperoxide induces the production of TG by improving substrate availability deriving from glycolysis rather than by stimulation of enzymatic machinery of lipogenesis. This notion can explain the fact that levels of both TG and cholesterol were increased.

The opposite effect on PFK and ACC activities could be explained by multiple actions of ascorbylperoxyde such as on the allosteric modulation by different molecules or by an upstream common effect such as on insulin. This last possibility seems to better be appropriate since it is more probable that ascorbylperoxide acts on only one system. For instance, insulin is known to stimulate the PFK activity by phosphorylation (Muller et al, 1988) whereas it activates ACC by dephosphorylation (Witters et al. 1988) or by stimulating its gene transcription through SREBP1 (Tong, 2005). Hence, the insulin-mimic effect of ascorbylperoxide can explain our results on PFK and ACC activation (Figure 3C). The loss of activity and protein level of ACC in animals infused with ascorbylperoxide can be explained by a negative feed-back effect of newly synthesized fatty acids on the expression of SREBP1 (Field et al., 2002). This last suggestion is supported by the negative correlation between ACC activity and plasma TG (Figure 4). On the other hand, it is not excluded that ascorbylperoxide

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can perturb the clearance of plasma lipids; this possibility was not an aim of the present study. The relation between ascorbylperoxide, insulin and clearance should be investigated in a further study in which all constituents of parenteral nutrition could be present to mimic more closely the clinical situation. Indeed, the generation of ascorbylperoxide in vitro is affected by the presence of others compounds: 1% multivitamin preparation in solution containing only dextrose (8%) generates 150 ± 11 μM ascorbylperoxide whereas the same concentration of multivitamin but in a completed parenteral nutritive solution, including amino acids and lipids, the ascorbylperoxide concentration is 36±1 μM (personal communication).

The relation between infused ascorbylperoxide and its concentration in urine is strong (Table 1). The high ratio of the urinary concentration on the quantity infused suggests that infused ascorbylperoxide is easily eliminated in urine. The study of the relations (figure 6) between urinary concentration of ascorbylperoxide and plasma TG, hepatic activity of PFK and ACC suggests that an efficient threshold did not exist for this molecule. The fact that ascorbylperoxide is detectable in urine of animals which were not infused with the molecule suggest an endogenous formation. That was also observed in our previous study (Knafo et al., 2005). The values of the endogenous threshold (0.8 μmol/mg creatinine) correspond to the inflexion point of the curves shown in Figure 6 (vertical dashed line). Therefore, a little addition of ascorbylperoxide could have metabolic consequences.

The premise of the study was to bring an explanation to the clinical observation done in premature infants that the absence of adequate photo-protection of parenteral nutrition was associated with a higher plasma TG compared to those receiving light-protected parenteral nutritive solution (Khashu et al., 2009). Since the light-exposure of parenteral nutritive solution favors the generation of ascorbylperoxide (Lavoie et al., 2004; Knafo et al., 2005),

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the present study suggests that the active agent coming from parenteral nutrition is the ascorbylperoxide, which induces a metabolic phenotype similar to an antenatal profile of the metabolism of glucose and lipids. Indeed, during the antenatal period, the activity of PFK is higher (Casado et al, 1996) and that of ACC is lower (Walther et al, 1995) than those after birth. The consequences to maintain such metabolism are unknown. However, because ascorbylperoxide derived from ascorbate, a vitamin largely available, further studies are needed to assess the biological or toxic role of this compound in human population.

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Footnote

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Legends For Figures

Figure 1. Plasma and hepatic triacylglycerol and cholesterol levels in function of infused amount of ascorbylperoxide. Triacylglycerol concentration in plasma (A) was significantly higher in animals infused with at least 4 µmol ascorbylperoxide per day whereas there was no difference in triacylglycerol between groups in liver (B). Total cholesterol concentration in plasma (C) was significantly higher in animals infused with at least 1 µmol ascorbylperoxide per day whereas there was no difference in cholesterol between groups in liver (D). Results are expressed as mean ± s.e.m. (n= 4-7). *: $p<0.05$; **: $p<0.01$.

Figure 2. Hepatic acetylCoA carboxylase in function of infused amount of ascorbylperoxide. In function of increased amount of ascorbylperoxide infused, the basal (A) as well as the total activity (B) of acetylCoA carboxylase (ACC) decreased, the ratio of the basal on the total activity (C), reflecting the proportion of un-phosphorylated and active form of the enzyme, increased whereas the relative protein amount (D) decreased; the vinculin abundance did not vary statistically between groups. Results are expressed as mean ± s.e.m. (n= 4-7). *: $p<0.05$; **: $p<0.01$.

Figure 3. Hepatic glucokinase and phosphofructokinase in function of infused amount of ascorbylperoxide. Glucokinase (GK) activity (A) was lower in the group receiving the highest amount of ascorbylperoxide whereas the phosphofructokinase (PFK) activity (B) was higher in animals infused with at least 4 µmol/d of ascorbylperoxide. The relative protein levels of GK (C) and PFK (D) as well as the reference protein vinculin were statistically similar between groups. Results are expressed as mean ± s.e.m. (n= 4-7). *: $p<0.05$; **: $p<0.01$.

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Figure 4. Hepatic acetylCoA carboxylase activity in function of the plasma triacylglycerol concentration. The acetylCoA carboxylase activity was negatively correlated (r^2 = 0.37, p <0.01) with plasma triacylglycerol concentration (y= -7.6 nmol•min^{-1}•mg prot^{-1}•mM^{-1}•x + 6.8 mM).

Figure 5. Hepatic markers of oxidative stress in function of infused amount of ascorbylperoxide. Redox potential of glutathione (A) as well as the level of isoprostane (B) were higher in animals infused with the highest amount of ascorbylperoxide. Results are expressed as mean ± s.e.m. (n= 4-7). *: p<0.05; **: p<0.01.

Figure 6. Plasma triacylglycerol concentration, hepatic phosphofructokinase and acetylCoA carboxylase activities in function of urinary triacylglycerol concentration. Triacylglycerol concentration (TG) in plasma (A) and phosphofructokinase (PFK) activity (B) increased positively in a logarithmic function with ascorbylperoxide in urine (TG: y= 0.063ln(x)+0.20 mM, r^2 = 0.69, p<0.01; PFK: y= 1.1ln(x)+5.7 nmol•min^{-1}•mg prot^{-1}, r^2 = 0.26, p<0.05). In contrast, the logarithmic relation between acetylCoA carboxylase (ACC) activity (C) and urinary ascorbylperoxide was negative (y= -0.7ln(x)+5.4 nmol•min^{-1}•mg prot^{-1}, r^2 = 0.50, p<0.01). Dashed line represents the calculated threshold limit of endogenous generation of ascorbylperoxyde in urine.

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Table 1. Ascorbylperoxide

Infused † (µmol / d)	Urine †† (µmol / mg creat.)	Urine †† (µmol / mL)	Urine / infused ††† (%)
- (n = 6)	0.5 ± 0.1	0.04 ± 0.01	-
1.1 ± 0.1 (n = 7)	1.5 ± 0.3	0.15 ± 0.03	53 ± 10
4.0 ± 0.1 (n = 6)	3.2 ± 0.7	0.30 ± 0.01	82 ± 7
7.0 ± 0.2 (n = 4)	6.7 ± 0.8	0.43 ± 0.05	87 ± 1

†: Ascorbylperoxide infused = mean of three days; ††: urine from last day; †††: ratio obtained after deduction of endogenous content (95% confidence value from group without infusion of ascorbylperoxide = 0.80 µmol/ mg creatinine or 54 µM). Urinary concentration of creatinine did not vary between groups (78 ± 6 µg/mL; $F_{(1,19)}<1.4$). Results are expressed as mean ± s.e.m.

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Table 2. Glutathione status

	Ascorbylperoxide infused (µmol / d):			
	0	1.1 ± 0.1	4.0 ± 0.1	7.0 ± 0.2
GSH (nmo/mg prot)	35 ± 5	82 ± 23	33 ± 13	19 ± 8 *
GSSG (nmol/mg prot)	0.35 ± 0.03	1.02 ± 0.29 *	0.37 ± 0.10	0.26 ± 0.06
GSSG / total glutathione (%)	2.2 ± 0.4	2.9 ± 0.6	3.0 ± 0.9	3.5 ± 1.1

Hepatic GSH level was significantly lower in the 7 µmol/d group (*: $p<0.05$), whereas GSSG level was higher in the 1.1 µmol/d group (*: $p<0.05$). There was no statistic difference between groups for GSSG / total glutathione (2 x GSSG / [GSH + 2xGSSG]). Results are expressed as mean ± s.e.m. (n = 4-7).

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Figure 1

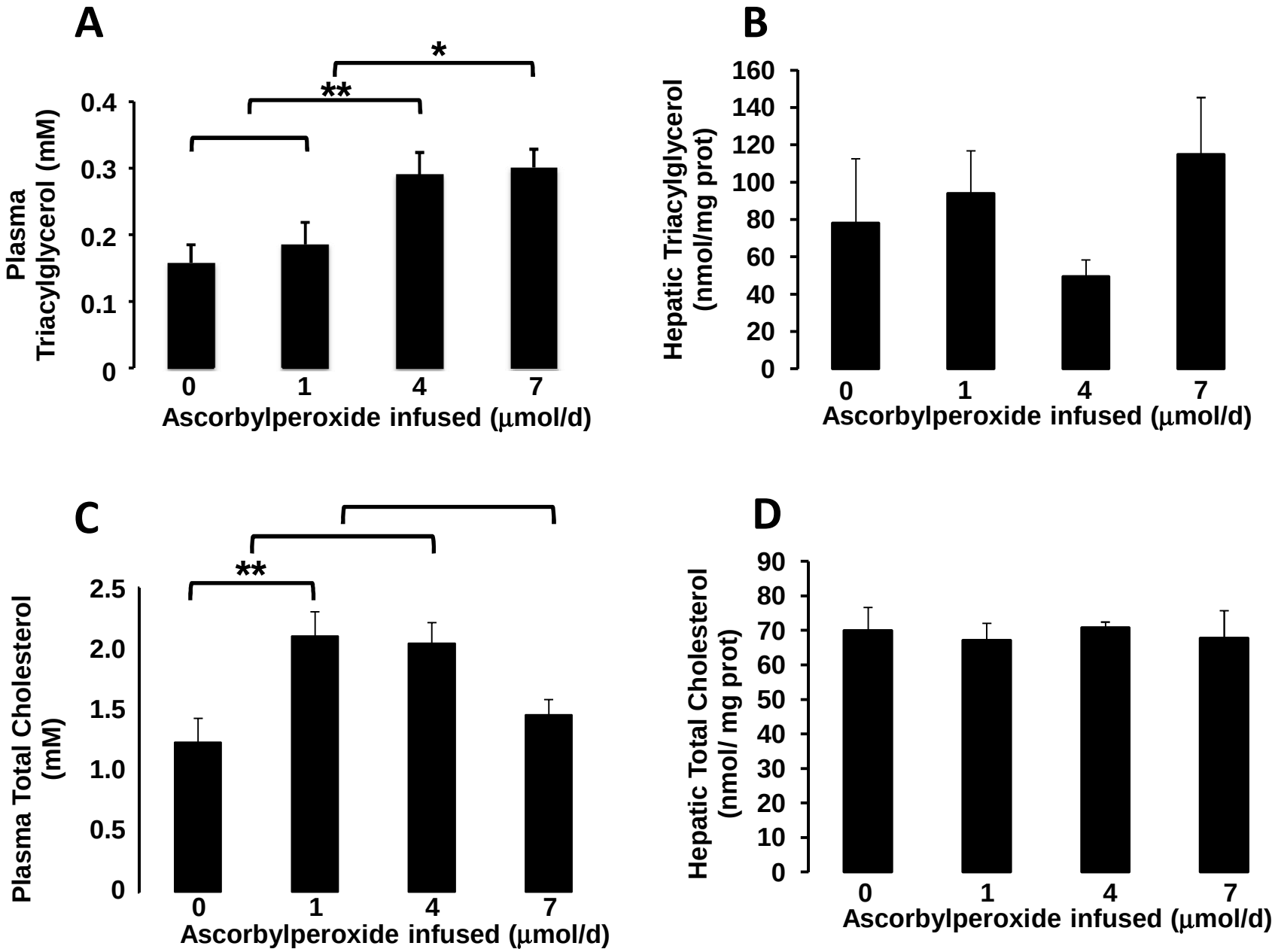

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Figure 2

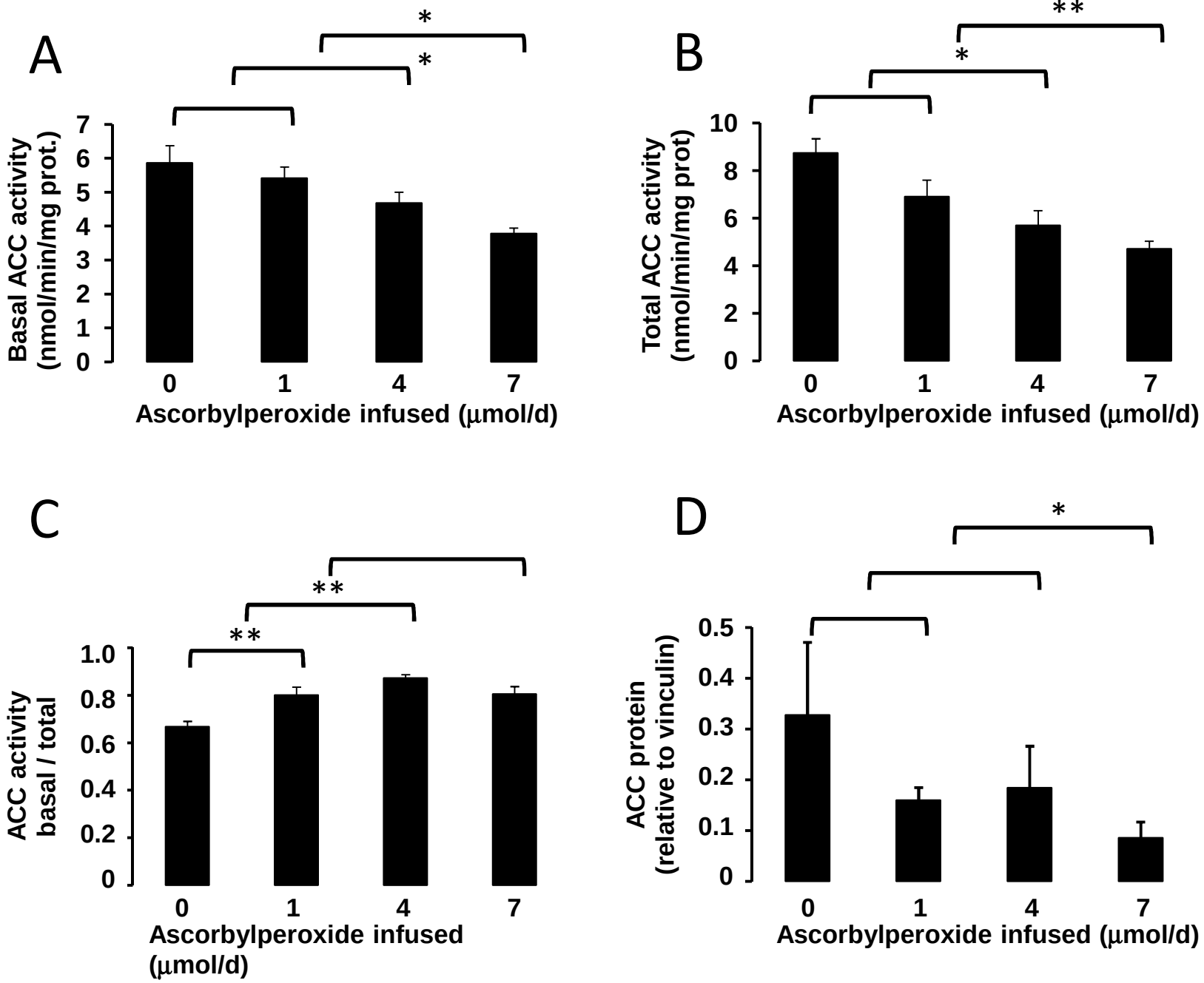

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Figure 3

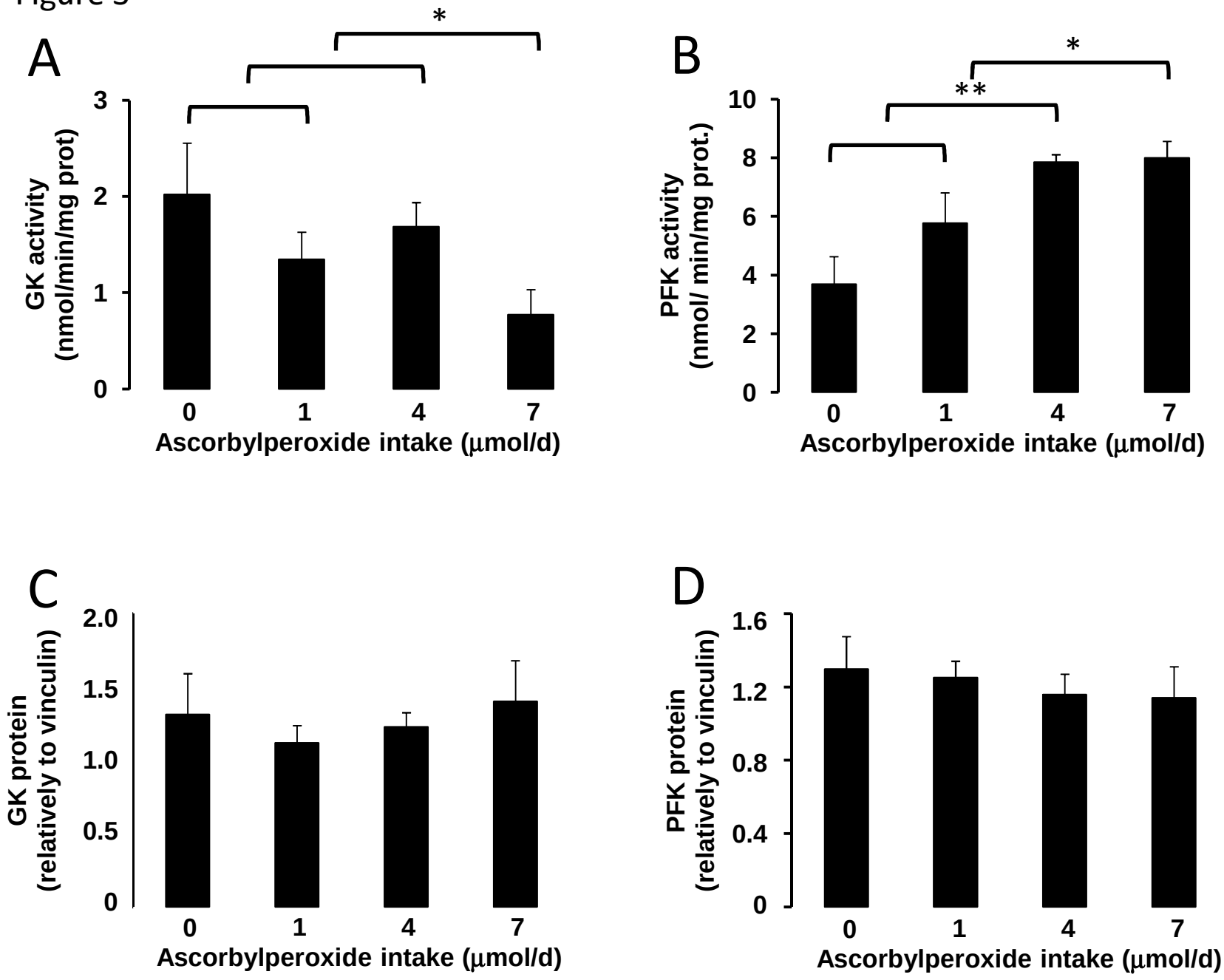

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Figure 4

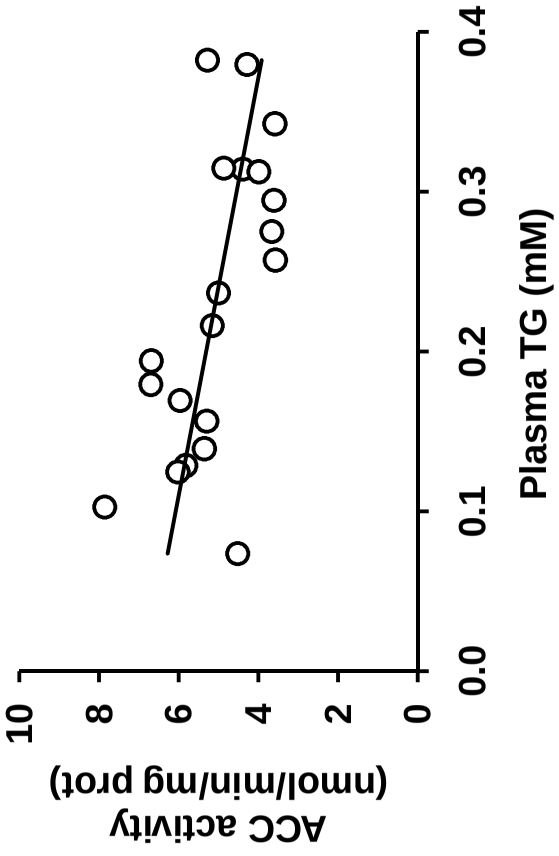

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Figure 5

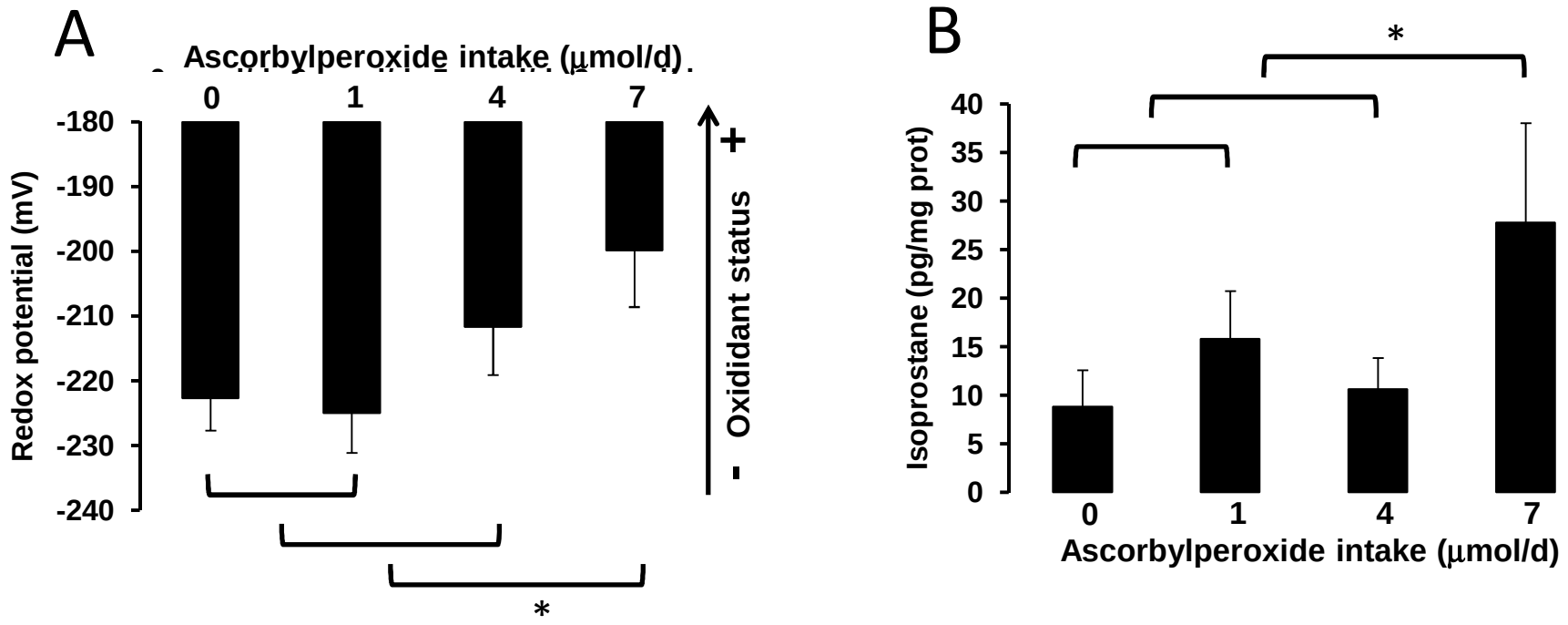

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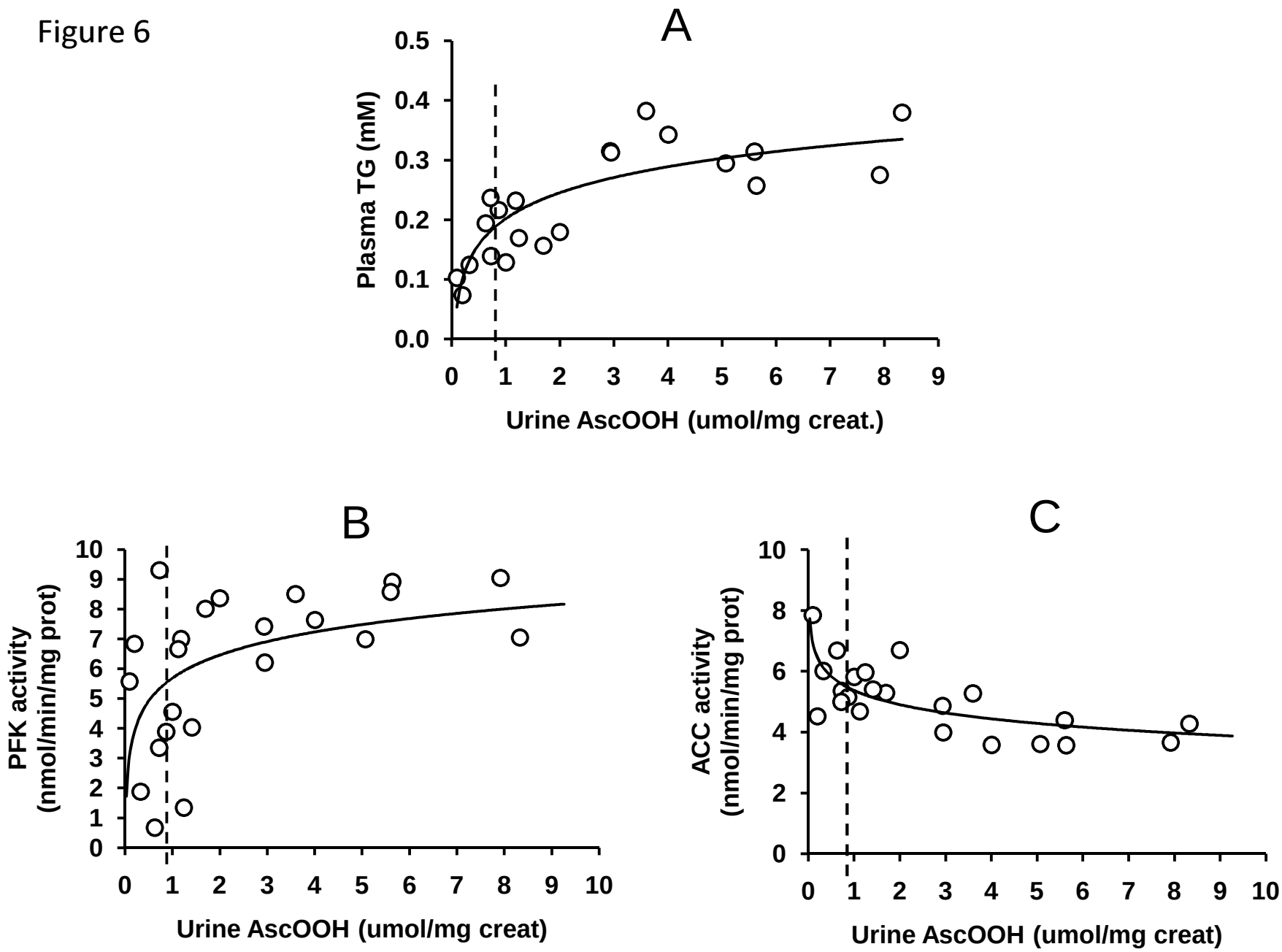
Figure 6



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