Photooxidation of Parenteral Multivitamins Induces Hepatic Steatosis in a Neonatal Guinea Pig Model of Intravenous Nutrition

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ABSTRACT

Photooxidation of multivitamin solutions results in the generation of peroxides. Because peroxides are associated with hepatic steatosis and fibrosis as well as cholestasis, we questioned whether multivitamins are implicated in hepatic complications of parenteral nutrition. Guinea pig pups were assigned to groups receiving intravenously either total parenteral nutrition, photo-protected or not, or a control solution (5% dextrose + 0.45% NaCl) supplemented with either a) multivitamins; b) photo-protected multivitamins; c) multivitamins without riboflavin; or d) peroxides (H_2O_2 , tert-butylhydroperoxide). After 4 d, liver was sampled for histology and isoprostane- $F_{2\alpha}$ levels, a marker of radical attack. Multivitamins as well as total parenteral nutrition were associated with steatosis (scored 0–4), the severity of which was reduced ($p < 0.05$) by photo protection. Although H_2O_2 is the major peroxide contaminating multivitamins, it did not induce steatosis scores different than the controls. Compared with controls, hepatic isoprostane- $F_{2\alpha}$ content increased in animals infused with H_2O_2 ($p < 0.05$), but not in those infused with Multi-12 pediatric multivitamins or total parenteral

nutrition. Results suggest that peroxides and/or free radicals are not mediators of the induction of steatosis observed with infusion of photo-exposed multivitamins, as there was no correspondence between histologic findings and hepatic levels of isoprostanes. It is suspected that a component of the multivitamin solution becomes hepato-toxic after photo-exposure, as indicated by the protective effect observed when withdrawing riboflavin. Photo-oxidation of multivitamins might be the common link between reports involving amino acids, lipids, and light exposure in the etiology of hepatic complications of parenteral nutrition. (*Pediatr Res* 52: 958–963, 2002)

Abbreviations

C, control
CVI, Cernevit multivitamins
MVP, Multi-12 pediatric multivitamins
MVI, pediatric multivitamins
TBH, tert-butylhydroperoxide
TPN, total parenteral nutrition

Exposure of TPN solutions to light is linked to alterations in hepatobiliary function and histology in a perfused liver model (1) as well as *in vivo* (2). In the presence of light, the parenteral multivitamin preparation is the site of reactions between oxygen and electron donors, resulting in the generation of peroxides. Hydrogen peroxide (H_2O_2), which is regarded as a key element in the oxygen toxicity of the cell, represents over 80% of peroxides generated in multivitamins (3). Organic peroxides have also been reported to contaminate solutions of parenteral nutrition (3–6). In parenterally fed neonates, the exposure of multivitamins to daylight is associated with an infused perox-

ide load and its urinary excretion (7). Peroxides have been linked to hepatic steatosis (8) and fibrosis (9), as well as cholestasis (10). Multivitamins and peroxides induce the same oxidant response in the lungs of guinea pig pups (11). The relative liver weight of these animals was 40% higher than controls (12), and histology revealed the presence of hepatic steatosis. We hypothesize that multivitamins might account for part of the role of light in TPN-related liver complications.

Because of the catalytic role of photo-excited riboflavin (13), protection of TPN solutions from exposure to light results in a decreased generation of peroxides and a reduction in their rate of infusion (3). As H_2O_2 is a weak oxidant, it can accumulate to high concentrations owing to its stability, or it can also be converted into reactive species (14) in the presence of transition metals (15, 16) such as Fe^{2+} and Cu^+ via the Fenton/Haber-Weiss reactions. Furthermore, the antioxidant components of the parenteral multivitamins would be expected to protect against the free radicals derived from peroxides generated in TPN solutions (12).

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The objective of this study was to evaluate the role of multivitamins in TPN-related liver complications as documented by histology and to separate the effect of light exposure from that of peroxides and/or free radicals.

METHODS

Animal model. Under ketamine/xylazine, 3-d-old guinea pig pups (Charles River, Montreal, Quebec, Canada) were fitted with a 0.4-mm polyurethane catheter (Luther Medical Products, Tustin, CA, U.S.A.) in the external jugular vein and exteriorized in the scapular region. The catheter was connected to a flow-through system permitting mobility of the animals, which were housed separately in plastic boxes with wire mesh bottoms. The animals were kept in a thermo-controlled environment with a 12-h light/dark cycle, and they were fed exclusively intravenously at 240 mL/kg/d. After 4 d, the animals were killed and the liver was divided into aliquots with samples kept in formol for histology or minced and stored at -80°C until analysis of triglycerides and isoprostanes, an index of radical attack (17). The study was performed in accordance with the guidelines of the institutional animal committee.

This animal model was chosen because of our previous unpublished observation of hepatic steatosis in guinea pig pups. To verify whether this steatosis could be prevented by photoprotection, the same experimental conditions had to be replicated.

Protocols. To separate the effect of multivitamins and light, animals were infused with one of the following regimens containing a multivitamin solution at a concentration similar to the one used in neonatal parenteral nutrition. Photoprotection was achieved by preparing the solutions in darkness and covering the infusion set with opaque material and using photo-protected intravenous tubing (13) provided graciously by Baxter Canada.

MVP + light: 1% (vol/vol) MVP (Sabex, Boucherville, Quebec, Canada) unprotected from ambient light, diluted in the control solution (50 g/L dextrose + 4.5 g/L NaCl + 1 unit heparin/mL); 5 mL of MVP contains the following: vitamin A, 2300 IU; vitamin D, 400 IU; α -tocopherol, 7 IU; vitamin K, 200 μg ; ascorbate, 80 mg; thiamine, 1.2 mg; riboflavin, 1.4 mg; pyridoxine, 1.0 mg; pantothenate, 5 mg; folate, 0.14 mg; cyanocobalamin, 1 μg ; nicotinamide, 17 μg ; biotin, 20 μg ; and the additives mannitol and polysorbates as emulsifiers.

MVP - light: 1% (vol/vol) MVP photo-protected, diluted in the control solution.

TPN + light: Two solutions unprotected from ambient light were infused in a "piggy-backed" set-up mixed close to the infusion site: a) control + 9.6 g/kg/d amino acids (Travasol, Baxter Canada, Mississauga, Ontario, Canada) + 2% MVP at 120 mL/kg/d; b) control + 9.6 g/kg/d amino acids + 7.6 g/kg/d lipids (Intralipid 20%, Pharmacia, Peapack, NJ, U.S.A.) at 120 mL/kg/d; for a final concentration (at 240 mL/kg/d) of 1% MVP + 4.8 g/kg/d amino acids + 3.8 g/kg/d lipids. The composition of the TPN regimen was derived from recommendations for studies in adult guinea pigs (18).

TPN (-) light: The same TPN preparation protected from ambient light.

To evaluate whether the effects observed with MVP and TPN were related to peroxides and/or free radicals, other groups of animals were infused with one of the following solutions:

C (control): 50 g/L dextrose + 4.5 g/L NaCl + 1 unit heparin/mL.

H₂O₂: control + 200 or 500 μM H₂O₂ (Aldrich Chemical, Milwaukee, WI, U.S.) (208 ± 15 and 500 ± 20 μM measured).

TBH: control + 50 μM TBH (Aldrich Chemical) (47 ± 1 measured).

To isolate the role of riboflavin and to determine whether the effects of multivitamins were specific to MVP, other groups of animals were infused with one of the following regimens:

C: same as above

MVP + light: as described above in protocol 1.

MVP + light without riboflavin: 1% MVP prepared by Sabex without riboflavin, unprotected from ambient light and diluted in the control solution.

MVI + light: 1% (vol/vol) MVI (Aventis, Strasbourg, France) unprotected from ambient light and diluted in the control solution; 5 mL solution reconstituted from powder contains the following: vitamin A, 2300 IU; vitamin D, 400 IU; α -tocopherol, 7 IU; vitamin K, 200 μg ; ascorbate, 80 mg; thiamine, 1.2 mg; riboflavin, 1.4 mg; pyridoxine, 1.0 mg; pantothenate, 5 mg; folate, 0.14 mg; cyanocobalamin, 1 μg ; nicotinamide, 17 mg; biotin, 20 μg ; and the additives BHA, BHT, and mannitol, as well as polysorbates as emulsifiers.

CVI + light: 1% (vol/vol) Cernevit (Baxter Canada) unprotected from ambient light and diluted in the control solution; the 5 mL solution contains vitamin A, 3500 IU; vitamin D, 220 IU; α -tocopherol, 11.2 IU; ascorbate, 125 mg; thiamine, 3.51 mg; riboflavin, 4.14 mg; pyridoxine, 4.53 mg; pantothenate, 17.25 mg; folate, 414 μg ; cyanocobalamin, 6 μg ; nicotinamide, 46 mg; biotin, 69 μg ; and the additives glycocholic acid, and soy bean lecithin.

Histology. After staining with hematoxylin-eosin, hepatic histology was performed, and the severity of steatosis scored 0–4 (Fig. 1) by a pathologist (P.B.) and a hepatologist (F.A.) unaware of the intravenous regimen received. The scores indicated the following: 0 = normal hepatic structure; 1 = presence of visible fat vacuoles in single, isolated hepatocytes; 2 = focal fatty changes of heterogeneous lobular distribution; 3 = diffuse fatty changes with rare "fat-free" zones; 4 = diffused fatty changes, without "fat-free" zones.

Analytical procedures. Peroxides were measured with the xylenol orange technique (19), as described previously. This chemical assay, which measures H₂O₂ as well as organic peroxides (19, 20) was validated for TPN solutions using an enzymatic assay (21). The mean peroxide levels for three samples were MVI = 295 ± 8 μM ; CVI = 358 ± 28 μM ; MVP = 198 ± 14 μM ; MVP photo-protected = 131 ± 10 μM ; and MVP without riboflavin = 129 ± 3 μM . Isoprostane F_{2 α} was measured using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). Liver triglyc-

HEPATIC HISTOLOGY (x250)**Steatosis
scored 0 to 4** **0 / 4** **2 / 4** **4 / 4**

Figure 1. Hepatic histology ($\times 250$) illustrating scores of severity of steatosis scored from 0 to 4.

eride levels were measured enzymatically using commercial kits (Roche Diagnostics, Laval, Quebec, Canada).

Statistical analysis. All results are presented as mean $\pm$ SEM. Results for steatosis were analyzed by Mann-Whitney test for nonparametric data. Anthropometric characteristics of the animals (Table 1) and hepatic isoprostane $F_{2\alpha}$ were compared by ANOVA after transformation (natural logarithm) to ensure homoscedasticity as determined by Bartlett's chi-square. The level of significance was set at $p < 0.05$.

RESULTS

There was no difference in the initial body weight of the animals within the various protocols (Table 1). In the first protocol, photo-protection was associated with a significantly lower ($p < 0.05$) relative liver weight (percentage of body weight).

Animals receiving photo-exposed multivitamin solutions infused as an admixture to C or to TPN presented varying degrees of hepatic steatosis. In cases of hepatic steatosis scored 2/4, the macrovesicular fatty changes were mainly observed in lobular zones 3 and 2. In specimens evaluated as showing scores of 3 to 4/4, nodules of fat-laden hepatocytes gathered into a circumscribed nodule. Whatever the steatosis score recorded, no inflammatory infiltration or enlargement of portal tracts were observed. Multivitamins as well as TPN were associated with steatosis (scored 0–4), the severity of which was reduced ($p < 0.05$) by photo-protection (Fig. 2A). Compared with MVP, the apparently lower score of hepatic steatosis observed in animals receiving TPN regimens did not reach statistical significance. These histologic findings were not accompanied by corresponding changes in hepatic isoprostane levels (Fig. 2B). Animals receiving photo-protected solutions

Table 1. Anthropometric characteristics of the animals

Intravenous solutions	No.	Initial body weight (g)	Change in body weight (%)	Relative liver weight (% body weight)
<i>Protocol I</i>				
MVP - light	9	116 ± 6	-15 ± 3	3.1 ± 0.1
MVP + light	9	120 ± 4	-19 ± 2	3.4 ± 0.1
TPN - light	6	124 ± 7	-15 ± 2	3.4 ± 0.2
TPN + light	6	128 ± 6	-15 ± 2	3.9 ± 0.2
<i>Protocol II</i>				
C	10	117 ± 6	-22 ± 3	3.2 ± 0.1
TBH 50 μ M	4	121 ± 10	-11 ± 4	3.3 ± 0.1
H ₂ O ₂ 200 μ M	6	115 ± 1	-19 ± 3	3.4 ± 0.1
H ₂ O ₂ 500 μ M	8	118 ± 5	-19 ± 3	3.2 ± 0.1
<i>Protocol III</i>				
C	10	107 ± 7	-25 ± 4	3.4 ± 0.1
MVP + light	9	109 ± 4	-13 ± 2	3.3 ± 0.1
MVP + light - riboflavin	9	129 ± 2	-15 ± 2	3.4 ± 0.1
CVI + light	11	101 ± 3	-9 ± 2	3.1 ± 0.3
MVI + light	5	114 ± 4	-11 ± 3	3.1 ± 0.3

Results are expressed as mean $\pm$ SEM; change in body weight occurred over the 4 d of the protocol. C, control = 0.45% NaCl + 5% dextrose; MVP, 1% Multi-12 Pediatric; MVI, 1% MVI Pediatric; CVI, 1% Cernevit; (+) light, exposed to ambient light; (-) light, photoprotected.

Figure 2. Effect of parenteral multivitamins (MVP), TPN (5% dextrose + 0.45% NaCl + amino acids + lipids + MVP) and light exposure ($\pm$ light) on (A) the severity of hepatic steatosis (scored 0–4) and (B) hepatic isoprostane F_{2α}. Multivitamins and TPN were associated with steatosis, the severity of which was reduced by photo-protection (- light). Photo-protection decreased significantly isoprostane levels, which did not correspond with the severity of steatosis as TPN induced significantly higher levels of this eicosanoid marker of free radical attack. Each bar represents the mean $\pm$ SEM (number of samples). * p < 0.05, ** p < 0.01.

had significantly lower isoprostane levels (p < 0.01). However, compared with MVP, those animals receiving TPN exhibited significantly higher (p < 0.01) hepatic levels of this eicosanoid marker of radical attack.

Figure 3, A and B, illustrates that the oxidant load induced by peroxides generated in light-exposed multivitamins or TPN did not account for the induction of hepatic steatosis. H₂O₂ infused at concentrations found in MVP or TPN (3) did not induce a score of steatosis significantly higher than the controls (Fig. 3A), which exhibited a lower score (p < 0.01) than animals receiving MVP exposed to light (Fig. 4). Results obtained with TBH infused at a concentration compatible with levels of organic peroxides detected in MVI (3) were similar to C and H₂O₂. The results presented in Figure 3B support the conten-

Figure 3. Effect of infused H₂O₂ and TBH on (A) the severity of steatosis and (B) hepatic isoprostane F_{2α}. H₂O₂ and TBH infused in a range of concentrations generated in multivitamins did not induce scores of hepatic steatosis higher than the controls (C = 5% dextrose + 0.45% NaCl). H₂O₂ induced an oxidant load, as it had a positive effect on levels of isoprostane F_{2α}. Each bar represents the mean $\pm$ SEM (number of samples). * p < 0.05.

tion that the infused peroxides carried an oxidant load. Indeed, the levels of hepatic isoprostanes found in animals receiving H₂O₂ were significantly higher than controls (Fig. 3B) and between 50% and 100% higher than in animals receiving multivitamin solutions inducing a score of steatosis >2 (Fig. 2B).

When exposed to ambient light, the different multivitamin solutions evaluated in this study (MVP, MVI, CVI) induced a similar score of hepatic steatosis, which was significantly higher (p < 0.01) than the score recorded in C or in the animals receiving MVP without riboflavin exposed to light (Fig. 4). The latter two groups exhibited a similar low score of hepatic steatosis.

Hepatic steatosis was confirmed by documenting higher (p < 0.05) liver triglyceride levels (0.81 ± 0.32 versus 0.23 ± 0.07 mg/mg protein) in a subgroup of animals with overt

Figure 4. Compared with the control (C = 5% dextrose + 0.45% NaCl), the photo-exposed (+ light) multivitamin preparations (MVP, MVI, CVI) enhanced hepatic steatosis (scored 0–4). The role of riboflavin on the effect induced by light exposure was tested using a preparation devoid of this photo-excitable vitamin (MVP without riboflavin). Each bar represents the mean $\pm$ SEM (number of samples). * $p < 0.05$, ** $p < 0.01$.

steatosis (score 2–4: 3.1 ± 0.3 , $n = 6$) compared with those with no or little steatosis (score 0 or 1: 0.7 ± 0.1 , $n = 13$).

DISCUSSION

Steatosis is a hepatic micro- or macrovesicular fat accumulation associated with metabolic diseases, diabetes, obesity (22), drugs and toxins (23), surgical procedures, and parenteral nutrition (21), as well as nonalcoholic steato-hepatitis, in which case an oxidant stress is thought to play a pathogenic role (24). TPN-related hepatic dysfunctions (25) have been linked to oxidant stress (26, 27) as well as genetic (28, 29), nutritional, environmental, and inflammatory factors (23). More specifically, the following nutritional factors have been associated with steatosis and/or cholestasis: enteral starvation (30, 31), an amino acid imbalance (32, 33), photo-oxidized products of amino acids (11, 12, 34), an excessive infusion of glucose (35) or lipids (36), a carbohydrate to nitrogen imbalance (37, 38), and the source of infused lipids (39).

The results of the present study suggest that TPN, when exposed to light, induces hepatic steatosis. The fact that this effect of light is linked to MVP points toward an oxidant stress (11, 40). The etiology of this steatosis might be explained by infused oxidants increasing lipogenesis, or decreasing lipid transport (2) and oxidative metabolism (41, 42). However, the results suggest that peroxides and/or free radical are not mediators of the induction of steatosis observed with infusion of

photo-exposed multivitamins, as there was no correspondence between histologic findings and hepatic levels of isoprostanes.

Starvation might account for the hepatic steatosis observed when glucose is the only source of energy provided with MVP. TPN prevented only partially the steatosis induced by MVP. This effect of TPN could be explained by the lipid emulsion acting either as a shield protecting the multivitamins against the effect of light or as a substrate improving the nutritional status. Since the effect of light was also observed with TPN, the later explanation is more likely. Among the nutritional factors contributing to steatosis, the carbohydrate to nitrogen imbalance might be implicated. However, light protection reduced the risk of steatosis even in animals receiving only dextrose + MVP. In rats, diets without choline are used to induce steatosis (43) and in humans, steatosis is corrected by giving choline (44). Choline is an important component of phosphocholine, which is involved in lipid transport. Therefore, the 99 mg% phosphocholine provided to the guinea pigs by the lipid emulsion might account for this observation.

Various sources of multivitamins (MVP, MVI, and CVI) were found to induce the same score of steatosis despite differences in vitamin concentrations, in additives (polysorbates, BHT, BHA, mannitol), in means of conservation (powder *versus* liquid form) or in levels of H_2O_2 . This is further evidence that infused H_2O_2 is not implicated in this common hepatic complication of TPN. Although TBH was not found to be associated with steatosis, this does not preclude a possible role for further organic peroxides generated by photo-oxidation. It is suspected that a component of the multivitamin solution becomes hepato-toxic after light exposure as indicated by the protective effect observed when withdrawing riboflavin. Apart from the generation of peroxides, photo-excited riboflavin also produces strongly oxidizing triplets. A direct effect of activated riboflavin on the induction of steatosis cannot be excluded. Photo-oxidation of multivitamins might be the common link between reports involving amino acids (34), lipids (39), and light exposure (1, 2) in the etiology of hepatic complications of parenteral nutrition.

Multivitamins and H_2O_2 induced similar responses on markers of oxidation measured in the lungs (11, 40), whereas in the liver they induced separate effects on hepatic isoprostane levels as well as on steatosis. After their infusion, multivitamins and peroxides will transit through the lungs before reaching the liver. This could explain in part why the responses elicited in these tissues are different. However, this discrepancy does not result from peroxides being consumed before reaching the liver, as they did cause a marked increase in hepatic isoprostane levels.

It is questionable whether our observations made in a 4-d infusion model can be extrapolated to subjects receiving complete TPN for a longer time, or to patients with low antiperoxide defenses such as premature infants (22). The development of hepatic steatosis observed in the present study could be related specifically to the animal model. Guinea pig tissues other than the brain do not use much glucose, and they do not respond to insulin by increasing glucose uptake. It is therefore important to recognize that interspecies differences make extrapolations to the clinical situation hazardous (21).

In summary, multivitamins exposed to light induce hepatic steatosis. This complication of TPN is not related to the level of hydrogen peroxide or to a radical attack. A photosensitive component of the multivitamin solution is suspected. In spite of its peroxide load, MVP carries antioxidant properties (12) as documented by the 3-fold lower hepatic isoprostane levels observed with photo-protected MVP (Fig. 2B) compared with C (Fig. 3B). Because free radicals are produced in response to various sources of oxidant stress, improving free radical scavenging properties of MVP by photo-protection (Fig. 2B) may in itself be of biologic significance.

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