Oxidative Stress in Children on Long-Term Parenteral Nutrition

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Abstract

Background: The immaturity of glutathione metabolism is believed to explain the oxidative stress observed in premature neonates on total parenteral nutrition (TPN). Animal studies report that peroxides contaminating TPN induce the low glutathione levels as a consequence of the inhibition of the generation of substrates for its synthesis. We hypothesize that older children receiving long-term TPN have low levels of glutathione with the consequence to have an increased oxidative stress.

Methods: Total glutathione and oxidative stress markers (hydroperoxides and isoprostane-F2α) were measured in urine, plasma or erythrocytes from 6 children aged 77 ± 24 months treated with TPN for a mean duration of 28 ± 7 months and from 7 control children aged of 87 ± 21 months.

Results: In the TPN group, glutathione was lower [p<0.01] whereas hydroperoxide and isoprostane-F2α were higher [p<0.01] than in control group. Plasma isoprostane-F2α was negatively correlated with erythrocytes glutathione [r² = 0.60, p<0.01]. Ascorbate levels were similar in erythrocyte, but higher [p<0.05] in urine of the TPN group.

Conclusion: Similar to premature infants, oxidative stress is increased in children on long-term TPN. The oxidative stress is associated with low glutathione.

ABBREVIATIONS
TPN: Total Parenteral Nutrition

INTRODUCTION

Total parenteral nutrition (TPN) is usually provided to patients with intestinal failure. This nutritional support is frequently used in infants born before 29 weeks of gestation due to their gastrointestinal tract immaturity. Although the TPN is essential for the growth of these neonates, it is an important source of oxidative stress as reported by previous studies [1-3]. In this population, this oxidative stress was associated with necrotizing enterocolitis [4], bronchopulmonary dysplasia [5-7] and retinopathy [4]. In animal, it was related to hepatic steatosis [8], glucose and lipid metabolism disorders [9], reduced pulmonary alveolar count [10] and initiation of fibrosis [11]. Few studies have investigated older patients to know whether TPN may be deleterious for health. The question is especially prominent in our pediatric hospital, for children on long-term home-TPN, who could benefit of the new development in TPN compounding for neonates [12].

The main source of oxidant molecules in TPN is multivitamin preparations [7,13]. The interactions between reductive nutrients such as ascorbate and dissolved oxygen [13,14] generate peroxides. During this process, ascorbate is oxidized into dehydroascorbate that is rapidly hydrolyzed into diketogulonate. The result is the loss of vitamin C, a powerful free radical scavenger, and the generation of oxidant molecules composed mainly with peroxide species, of which about 80% is H2O2 [13]. In presence of iron or copper, as present in TPN solutions, peroxides can be transformed in free radical species as predicted by the Fenton reaction. The negative impact of free radicals on DNA, lipids and proteins are well reported; thus, a loss of ascorbate may be deleterious for health. On the other hand, exposure to large amount of peroxides from TPN could lead to metabolic perturbations following oxidation of redox-sensitive thiols of specific proteins [15,16]. For instance, peroxides from TPN oxidize an essential thiol function of methionine adenosyltransferase, the first enzymes in transformation of methionine into cysteine, inhibiting its activity and consequently limiting the synthesis of glutathione [17]. Glutathione is an essential element of detoxification of peroxides, via glutathione peroxidase.

This metabolic action of peroxides from TPN is
highly suspected to explain the low level of glutathione reported in premature infants on TPN [18,19]. This vicious cycle leads to accumulation of peroxides, which can exert a toxic action directly on metabolism [i.e. thiol oxidation] or/and after their transformation into free radicals [i.e. following a Fenton-like reaction].

Because pediatric TPN solution contains multivitamin preparation (of which ascorbate), we hypothesize that older children receiving long-term TPN have low levels of glutathione and high indices of oxidative stress. Therefore, the aim of this study was to compare the presence of markers of oxidative stress (hydroperoxides, isoprostane-F$_{2\alpha}$), levels of glutathione and ascorbate in a group of children receiving long-term TPN, to a control group of children of comparable age.

**MATERIALS AND METHODS**

**Patients**

Thirteen children followed by the Department of Pediatric Gastroenterology and Nutrition of the Sainte-Justine Hospital [Montreal] were enrolled. On the day of inclusion, parental consent was signed, medical history and clinical data were noted, and blood [3ml] and urine samples were collected. The ethic committee of the institution has approved the study. For the TPN group, all children on TPN treatment were included except those with signs of infection and when parenteral solution was stable (unchanged for at least 4 weeks). Six female children older than 12 months of age and under TPN for 6 months were included. TPN was considered as exclusive when it represented more than 75% of total daily intake. Table 1 presents some nutritive characteristics of TPN. The control group was composed of seven children, matched for sex and age, without medical problems, but having a one-day stay at hospital for benign surgery or digestive endoscopy. Excluded from this control group were all those with a suspicion of inflammatory bowel disease, renal or hepatic failure, chronic intestinal pseudo-obstruction [three]. At the time of sampling, the child with chronic diarrhea had a yGT value 9 times higher than normal value. Subtle signs of liver damage [yGT and/ or ALT >2 normal value] were observed in three other patients. TPN was considered as exclusive when it represented more than 75% of total daily intake. Table 1 presents some nutritive characteristics of TPN. The control group was composed of seven children, matched for sex and age, without medical problems, but having a one-day stay at hospital for benign surgery or digestive endoscopy. Excluded from this control group were all those with suspicion of inflammatory bowel disease, renal or hepatic failure, metabolic disease, malnutrition or signs of infection.

**Samples preparation**

Aliquots of fasting blood [collected on EDTA] and urine were processed and stored at - 80°C within one hour of collection. Erythrocytes and plasma were separated by centrifugation at 7200 g for 4 minutes. For the determination of vitamin C [1], an aliquot of red blood cells was mixed with 5 volumes of 10% [w/v] meta-phosphoric acid; 2] in urine, one ml of supernatant of centrifuged urine [7200 g - 1 min] was acidified [pH 3.5- 4] with one ml of 0.1% [v/v] formic acid. For the quantification of 8-isoprostane F$_{2\alpha}$, 150 ml of plasma or 500 ml of centrifuged urine was mixed with one volume of buffer [50 mM Tris Base, 0.1 mM EDTA, 200 µM indomethacin, pH 7.4]. For all other assays, samples were aliquoted and directly frozen.

**Determinations**

Levels of blood glucose, plasma triacylglycerol and cholesterol were measured by the hospital biochemical service. The concentrations of hydroperoxide [H$_{2}$O$_{2}$ equivalent] in urine and in parenteral solutions [with in decreasing concentration of Multi-12 pediatrics [Sandoz, Boucherville, Qc, Canada]] were quantified by the ferric-orange xylenol [FOX] technique as previously described [20]. The quantities of free 8-isoprostane F$_{2\alpha}$ in plasma and total 8-isoprostane F$_{2\alpha}$ in urine were determined by using a competitive enzyme-linked immunoassay kit from Cayman Chemical [Ann Arbor, MI, USA] [8]. The quantification of vitamin C [ascorbic acid] in erythrocytes and in urine was measured by using an Ascorbic Acid Assay Kit [BioVision, Mountain View, CA, USA] as per vendor’s protocol. Total glutathione level, in erythrocytes, was quantified by using the method described by Griffith [21]. Glutathione peroxidase activity in erythrocytes was measured as previously described [22]. Total protein level was determined by using a commercial kit based on the Bradford method [Bio-Rad, Mississauga, Ont, Canada].

### Table 1: Nutritive characteristics of TPN.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TPN</th>
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<tbody>
<tr>
<td>Duration of TPN</td>
<td>28 ± 7 months</td>
<td>26 ± 7 months</td>
</tr>
<tr>
<td>Total caloric intake</td>
<td>85 ± 7 kcal/kg/d</td>
<td>87 ± 7 kcal/kg/d</td>
</tr>
<tr>
<td>TPN calories</td>
<td>1036 ± 165 kcal/d</td>
<td>1063 ± 165 kcal/d</td>
</tr>
<tr>
<td>TPN on total intake</td>
<td>75 ± 8 %</td>
<td>77 ± 8 %</td>
</tr>
<tr>
<td>Proportion of multivitamin (v,v) in TPN</td>
<td>0.33 ± 0.06 %</td>
<td>0.35 ± 0.06 %</td>
</tr>
<tr>
<td>Vitamin C from TPN</td>
<td>4.9 ± 1.2 mg/kg/d</td>
<td>4.8 ± 1.2 mg/kg/d</td>
</tr>
<tr>
<td>Copper</td>
<td>≤ 15 kg: 20 µg/kg/d</td>
<td>≤ 15 kg: 20 µg/kg/d</td>
</tr>
<tr>
<td>Iron</td>
<td>&gt; 15 kg: 300 µg/d</td>
<td>&gt; 15 kg: 300 µg/d</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>100 µg/kg/d</td>
<td>100 µg/kg/d</td>
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**Table 2: Anthropomorphic and biochemical status of children on TPN or not.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TPN</th>
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<tbody>
<tr>
<td>Number of individuals</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Age (month)</td>
<td>87 ± 21</td>
<td>77 ± 24</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>29.4 ± 4.0</td>
<td>18.8 ± 4.0</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>135 ± 18</td>
<td>103 ± 12</td>
</tr>
<tr>
<td>BMI (Body Mass Index)</td>
<td>18.0 ± 1.3</td>
<td>16.7 ± 0.3</td>
</tr>
<tr>
<td>Z score height / age</td>
<td>1.01 ± 0.03</td>
<td>0.89 ± 0.03*</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>5.4 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>Triacylglyceride (plasma) (mM)</td>
<td>0.63 ± 0.14</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>Total cholesterol (plasma) (mM)</td>
<td>4.0 ± 0.2</td>
<td>2.9 ± 0.2**</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>* p&lt;0.05; ** p&lt;0.01</td>
<td></td>
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**Statistical analysis**

The homogeneity of variances between groups was assessed by the Bartlett Chi$^{2}$ test. Values, reported as mean ± S.E.M. for isoprostane F$_{2\alpha}$, hydroperoxides and vitamin C in urine have been transformed by the natural logarithm to meet homoscedasticity. Student T-test was used to challenge the null hypothesis between control and TPN group. The significance was set at p < 0.05.
RESULTS

Table 2 shows characteristics of the children participating in the study. There was no difference between groups for age, body weight, height and BMI, whereas the height relative to the age of children fed intravenously was lower [p< 0.05] than that of controls. In the TPN group, total cholesterol concentration was lower [p< 0.01].

The Figure 1 confirms the link between the multivitamin preparation and peroxides that contaminating parenteral solutions. The concentration of peroxides increased in function of the multivitamin percentage and the time since the compounding of the solution. A plateau was reached in 3 hours with 0.75% multivitamin. With the mean proportion of 0.33% of multivitamin of the solution. A plateau was reached in 3 hours with 0.75% multivitamin percentage and the time since the compounding of solutions. Mean ± S.E.M. (for some value, the S.E.M was smaller than used symbol), n= 3 different bottles of Multi-12 pediatrics.

Compared to controls, the levels of both markers of oxidative stress [Table 3], hydroperoxides in urine and isoprostane F2α in plasma and urine, were higher [p< 0.01] in the TPN group. In contrast, glutathione levels in erythrocytes were lower [p<0.01] in the TPN group whereas the glutathione peroxidase activity was similar in both groups. In addition, urinary vitamin C concentration was higher [p<0.05] in children on TPN.

The urinary hydroperoxide concentrations [Figure 2] were positively dependent on the amount of vitamin C intravenously administered to the TPN group [slope of the linear relation = 64 [nmol/mg creatinine]/[mg/kg/d]; intercept = -69 nmol/mg creatinine; r² = 0.89, p<0.01]. Levels of isoprostane F2α in plasma as well as in urine were independent of the amount of vitamin C intravenously administered [r² < 0.03] [correlations not shown]. Plasma concentrations of isoprostane F2α correlated negatively with the erythrocyte glutathione levels [slope of the logarithmic relation = -1.1 ln[(mg/mg prot plasma) / (nmol/mg prot red cells)]; intercept = 3.0 ng/mg; r² = 0.60, p<0.01] [Figure 3]. Urinary concentrations of isoprostane F2α were not influenced by erythrocyte glutathione [r² = 0.03] [correlation not shown].

The results presented in Figure 4 suggest that the intravenous intake of vitamin C from TPN, was responsible for 90% of variation in urinary levels of vitamin C observed in the TPN group [slope of the linear relation = 1.9 [mol/mg creatinine] / [mg/kg/d]; intercept = -2.7 mol/mg creatinine; r² = 0.90, p<0.01]. The vitamin C level in red cells was not influenced by the variation in intravenous intake of vitamin C [r² = 0.06].

DISCUSSION

The main findings of the present study are that children receiving long-term TPN undergo strong oxidative stress [high levels of peroxide and isoprostane] and have low levels of glutathione whereas they are not deficient in vitamin C.

We expected to find elevated markers of oxidative stress in children on TPN because it is known that the interaction between vitamin C and dissolved oxygen in TPN generates high amount of peroxides [13,14] and loss of vitamin C [5,23,24]. The nice and positive correlation between urinary concentrations of peroxides in function of the amount of multivitamin or vitamin C received by the children confirms their chemical relation and is in agreement with previous findings done in premature newborns on TPN [13,14]. The fact that the urinary levels of peroxides strongly increase in urine suggests that children are unable to quench all peroxides infused with TPN [Figure 1]. During the process leading to the generation of H2O2, vitamin C is oxidized into dehydroascorbate, which is unstable in solution and is rapidly hydrolyzed into 2,3-diketogulonate, first step in its catabolism. This loss of vitamin C in TPN is well reported [5,25,26]. The half-life of vitamin C in TPN is well reported [5,25,26]. The half-life of vitamin C in TPN has been calculated to be between 3 to 10 hours depending on the temperature of conservation [5,27]. Although this destruction of ascorbate, our data suggests that it remains sufficient vitamin C to cover the needs of children. Indeed, the erythrocyte levels were similar in children on TPN and in controls; the values were in the normal range [28]. Furthermore, the children seem receive an excess of ascorbate as suggested by the positive correlation between their vitamin C intake and urinary levels.

<table>
<thead>
<tr>
<th>Table 3: Oxidant and antioxidant status of children on TPN or not.</th>
<th>Control (7)</th>
<th>TPN (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroperoxides in urine (nmol/ mg creatinine)</td>
<td>58 ± 14</td>
<td>245 ± 114**</td>
</tr>
<tr>
<td>Total Isoprostane F2α in urine (mg/mg creat)</td>
<td>1.0 ± 0.3</td>
<td>9.5 ± 4.4**</td>
</tr>
<tr>
<td>Free Isoprostane F2α in plasma (ng/mg prot)</td>
<td>1.0 ± 0.1</td>
<td>1.9 ± 0.3**</td>
</tr>
<tr>
<td>Total glutathione in erythrocytes (nmol/mg prot)</td>
<td>6.3 ± 0.5</td>
<td>3.0 ± 0.4**</td>
</tr>
<tr>
<td>Glutathione peroxidase in erythrocytes (nmol/min/mg prot)</td>
<td>0.44 ± 0.02</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Vitamin C in erythrocytes (mM)</td>
<td>62 ± 8</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Vitamin C in urine (nmol/mg creat)</td>
<td>1.0 ± 0.5</td>
<td>6.9 ± 2.8*</td>
</tr>
</tbody>
</table>
The high levels of isoprostane $F_{2\alpha}$ in both plasma and urine, which are associated with free radical injury, underline the presence of oxidative stress in children on TPN. Interestingly, the elevated concentration of vitamin C, which is a free radical scavenger, did not influence the generation of isoprostane in these children. The absence of prevention by vitamin C may be explained by the fact that this vitamin is hydro soluble whereas isoprostane is derived from peroxidation of membrane lipids. Does this mean that the transition metals, iron and copper present in TPN can induce the formation of radical species with affinity for membrane lipids? The current study cannot answer to this question. Plasma isoprostane was influenced [60% of its variation] by erythrocyte level of glutathione. This last observation suggests that the glutathione system is efficient to prevent the transformation of infused peroxides into free radicals, thus limiting the lipid peroxidation and generation of isoprostane.

Peroxides are detoxified by the action of glutathione peroxidase using glutathione as electron donor. In children on TPN, the activity of this enzyme is adequate but the level of glutathione is low compared to control group. The gold standard for determination of enzymatic activity is to do the assay in presence of saturate level of substrate or co-substrate, here glutathione. Thus, our results demonstrate that the levels of glutathione peroxidase in TPN group were similar to the control. However, in vivo, because the level of glutathione is low in TPN group, the activity of the enzyme is lower, limiting its capacity to detoxify peroxides infused with TPN. Peroxides are instable molecules easily transformed in free radical by a Fenton-like reaction. Thus, the in vivo level of glutathione seems to be a key element to prevent oxidative stress in this population. The low concentration of glutathione observed in TPN group can be explained by the presence of peroxides themselves. As underlined in Introduction section, TPN or high level of peroxides limits the synthesis of glutathione following inhibition of methionine adenosyltransferase [17], the first enzyme in the cascade responsible for the generation of cysteine from methionine. Because the availability in cysteine is a limiting step in glutathione synthesis, a result of this inhibition is a lower level of glutathione in liver and in blood [17].

The similarities between the oxidative status in children (present study) and premature newborns on TPN are striking. Both populations present low glutathione levels [18,19] and high oxidative stress markers such as isoprostane [5] and peroxide in urine [29,30]. This is surprising, considering that the TPN...
solution administered in these two populations present great differences, in both, the dose of multivitamins and the duration of this mode of nutrition. However, even though the multivitamin preparation added to the TPN solution of our patients [0.33 ± 0.06%, [v/v]] differed from that for premature infants [1 to 1.5%], these TPN solutions generated large amount of peroxides [from Figure 1, 0.33%; 288 ± 9 mM [n=3] and 1%; 433 ± 46 mM [n=3] in three hours]. In addition, premature infants received TPN for a mean time of 2 weeks, whereas children in the present study were administered TPN for 28 ± 7 months. Thus, the present study demonstrates that the oxidative stress induced by TPN is not specific to one population.

The long-term impact of TPN is mainly associated with liver diseases. In children, the long-term outcome of TPN is poorly documented. Peyret B et al. [31] reported that, in children on TPN since the age of 1.5 ± 0.5 year, the biochemical liver abnormalities appeared after 3 years on TPN. The subtle signs of liver injury [gGT and/or ALT > 2 normal value] observed in three children of this present study may be explained by a TPN duration of only 2.3± 0.6 years. Peyret B et al. [31] demonstrated that liver fibrosis, documented by biopsies, is dependent of the duration of TPN. Hepatic fibrosis was noted in 94% of children who were on TPN for a mean duration of 3.2 ± 0.9 years. Fibrosis is known to be inducible by products derived from lipid peroxidation such as aldehydes [31,32]. The high level of isoprostane in our TPN group testifies to lipid peroxidation. Infusion of TPN or H2O2 has been reported to stimulate in animals the expression of procollagen mRNA [11]; procollagen is frequently used as a marker of fibrosis development [33,34]. The addition of glutathione into infused solutions resulted in increased tissue glutathione levels and prevented the expression of mRNA of procollagen [1]. Because markers of lipid peroxidation [isoprostane] are high in TPN group compared to controls and because glutathione level measured in children on TPN was at about 50% of the level observed in control children, we suspect that oxidant molecules contaminating TPN may be one of the main inducer of hepatic diseases associated with TPN.

A limitation of this study resides in the low number of children included, in spite of the duration of two years for the recruitment. On the other hand, the large differences between groups in parameters measured demonstrated that a high statistical power was reached, which validates our results. However, we are aware that a confirmation of our results by another team would strengthen the universality of the concept. As a result of haphazard, only females were recruited. Knowing the sex dependency of antioxidant defenses, at least in premature infants [18,35,36], the comparison between groups with different proportion of males / females could be different. Premature male infants on TPN have lower cellular glutathione level [18,19]. Thus, our observations with female children could be exacerbated in males.

CONCLUSION

The originality of the study is that for the first time we have documented that, as currently administered, home-TPN for children is associated with a strong oxidative stress. The hypothesis explaining the observed oxidative stress is based on the presence of high level of peroxides in TPN. Metabolic explanations are based on animal and clinical studies [> 50% of references cited here]. However, it is not excluded that the nutritional quality by itself may play a significant role. Regardless of the sources of this oxidative stress, knowing the harms of this stress, it would be important for children’s health to prevent or correct it. By demonstrating a similarity of source and cause between these children and premature newborns, the study suggests that population of children on long-term home-TPN could benefit of new developments in TPN compounding for neonates [12].

FUNDING ACKNOWLEDGEMENT

Canadian Institutes for Health Research [NMD-98028].

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