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Phytosterols and Lack of Occurrence of Cholestasis in Rats Nourished Parenterally or Orally

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1. Introduction

Intravenous lipid emulsions (ILEs) have been marketed to infuse fat within total parenteral nutrition (TPN) in order to prevent essential fatty acid deficiency. Only recently, ILEs have been recognized to have therapeutic effects in gastrointestinal, cardiovascular, pulmonary, oncologic, autoimmune, and critical care diseases. At the same time, toxic substances such as phytosterols have been found in ILEs. Phytosterols have been related to a TPN-associated complication defined parenteral nutrition-associated cholestasis (PNAC) (1-5).

In a previous study (6), we found phytosterols to be considerably present in all the lipid emulsions analyzed so as to exceed the amount physiologically absorbed by the gut. In this study, we want to determine the plasma amount of phytosterols deriving from different oil sources in rats receiving parenteral nutrition, and compare it with those in rats on standard oral diet. Subsequently, we want to verify whether phytosterols have an impact on the occurrence of cholestasis. Finally, we intend to verify whether the addition of glutamine changes the outcomes.

Phytosterols, cholesterol, intravenous lipid emulsions, cholestasis, gas chromatography

ILEs: intravenous lipid emulsions
PNAC: parenteral nutrition-associated cholestasis
IL: parenteral nutrition with soy bean oil
TPN : total parenteral nutrition
ILG: parenteral nutrition with soy bean oil plus glutamine
CTR: standard control
ALT : alanine aminotransferase
ALKP : alkaline phosphatase

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GC : gas chromatography
SD: standard deviation

2. Materials and methods

2.1 Animal experiments

Animal and plant sterols were quantified in the plasma of fifteen 7-week-old genetically inbred male Wistar rats (Charles River, Calco, Como, Italy), divided in three groups: one group was infused with TPN containing Intralipid® (Fresenius Kabi AG, Germany) as lipid emulsion (IL), a second group received the same regimen with the addition of glutamine (15% of total amino acid composition) (ILG), and the third group was fed chow and served as standard control (CTR). PN was daily injected intraperitoneally for 5 days. Diets were isocalorical and their gross energy density was 10 kcal/die, divided into protein 20, lipid 40 and carbohydrate 40 in case of TPN and 5% fats (from soy), 20% proteins, and the remaining carbohydrates from chow. The animals, housed in individual room with controlled temperature and light conditions, had open access to food (Mucedola 2518; Settimo Milanese, Milan, Italy). They were allowed to acclimatize to these conditions for a week, then they were randomly divided into the three groups. Animal care, housing and killing met the guidelines of the Italian Health Ministry, which approved the study. Rats were weighted at the beginning of the study and before being killed. In the fifth day the animals were killed and their blood and liver samples were taken. Some blood samples were sent to the laboratory to determine liver function tests, lipid profile, and bile acids by enzymatic method, while others were collected in Eppendorf with EDTA and stored at – 80°C until analysis. Some liver samples (4–6 μm) were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin, and stained with hematoxylin and eosin prior to microscopic examination.

2.2 Reagents, solvents, and standards

Chloroform (analytical reagent grade), *n*-hexane (analytical reagent grade), methanol (Lichrosolv), diethyl ether, anhydrous sodium sulphate, potassium chloride, and potassium hydroxide were supplied by Merck (Darmstadt, Germany). Acetone (AnalaR®) was purchased from BDH (VWR International Ltd., Leicestershire, UK). Bidistilled water and silylating agents (pyridine, hexamethyldisilazane and trimethylchlorosilane) were supplied by Carlo Erba (Milano, Italy). (24*R*)-ethylcholest-5-en-3β-ol (β-sitosterol) purity: 60% β-sitosterol and 30% (24*R*)-methylcholest-5-en-3β-ol (campesterol) was purchased from Research Plus (Bayonne, NJ, USA). (24*S*)-Ethylcholest-5,22-dien-3β-ol (stigmasterol) (purity: 93%), cholest-5-en-3β-ol (cholesterol) (purity: 99%), and 5α-cholestane (purity: 97%) were purchased from Sigma (St. Louis, MO, USA). The purity of the standards was controlled by gas chromatography (GC).

2.3 Quantification of sterols

Lipid extraction and sterol determination were performed in plasma by a technique which processes were shown in tables 1.

Plasma levels were quantified ***by Gas chromatography-mass spectrometry*** technique according to ***Cold trapping technique,*** *"solvent vent injection"* mode.
Sample pretreatment ***Sample dilution:*** rat plasma sample 50 µl in sterile saline 50 µl (1:1 dilution). ***Internal standard solution:*** 5α-cholestane in ethanol 1.26 ± 0.06 mg/ml. ***Sterol standard solution:*** mixed of cholesterol/cholestanol/campesterol/stigmasterol/sitosterol in ethanol 2958 ± 0.6/384 ± 0.2/0.77 ± 231 ± 0.2/60 ± 0.2/302 ± 0.2 µg/300 µl respectively. ***Saponification*** and ***extraction procedure:*** sample 100 µl + "work" internal standard 1 ml (internal standard in diethylether/tetrametilammonium hydroxide/isopropanol (1:13:36 v/v/v) - *Incubation* at 80°C for 15 min - *Extraction* by tetrachloroethylene/methylbuthyrate 500 µl (1:3 v/v) and sterile water 2 ml. Organic phase exsiccation under nitrogen flow and derivatization with Tri-Sil® silyling mixture at 60°C for an hour.
Gas chromatography conditions ***Injection volume:*** extracted solution 2 µl. ***Gas chromatograph:*** GC HP 5890 Agilent with autosampler. ***Gas carrier:*** highly purified helium. ***Flow rate:*** 0.5 ml/min. ***Column conditions:*** capillary column HP-5MS, 5% PH SM Siloxane Crosslinked, heated from 100° C to 280° C for 21 min. ***Detector: mass spectroscope*** MS 5973 Agilent. ***Oven temperature:*** 60°C ***Software:*** HP 5973N, rev D002 for data registration, integration and treatment. ***Assay validation:*** specificity, detection and quantitation limit, linearity, precision and accuracy. Every sample was triple-tested.

Table 1. Determination of sterols

2.4 Data analysis

Results were summarized according to distribution in mean and standard deviation (SD) or median and range. Comparisons of means among experiments groups were carried out using a 1-tailed analysis of variance test with multiple-comparison tests using the Bonferroni option to compare plasma sterol contents among groups. Comparisons of medians were performed using the Kruskal Wallis and Wilcoxon test for non parametric data. Statistical tests were performed using the STATA Software 11 (Santa Monica, California). Then, the results were associated with the liver specimens, which were read by two different pathologists, both blinded to the type of nourishment received by the rats and unaware of each other's opinion. Statistical differences were considered significant at a p-value of <0.05.

3. Results

All animals behaved normally during the study. They all gained weights and there was non significant differences among groups during the study. Summary of descriptive data is summarized in table 2. Serum liver function tests were determined as marker of liver

alteration. Total and direct bilirubin, cholesterol, and biliary acid were averagely normal and they not differed among groups. Alanine aminotransferase (ALT) and alkaline phosphatase (ALKP) reached a statistically difference. Noteworthy, both had a higher level in the control group than the others' receiving TPN. Also glucose (not reached a significant difference), along with bile acids were increased in the control group as compared to the other two. Tryglicerides (p value= 0.05 borderline) were highly represented in the ILG group. Upon collection, livers were noted to be dark red in all groups and their weights were similar.

	IL	ILG	Control	p-value
Weight delta gr, (median/range)	20 (5-25)	15 (5-20)	20 (5-25)	NS
ALT U/L (median/range)	39 (25-69)	31 (13-41)	56 (45-201)	0.018
ALKP U/L (median/range)	338 (69-510)[A]	388 (276-426)[B]	578 (505-657)	0.016
Glucose mg/dl (median/range)	264 (233-312)	352 (278-407)	444 (303-464)	NS
Total bili mg/dl (median/range)	0.08 (0.07-2.55)	0.03 (0.03-0.03)	0.12 (0.06-0.17)	NS
Direct bili mg/dl (median/range)	0.02 (0.02-0-03)	0.045 (0.02-0-05)	0.04 (0.03-0.04)	NS
Bile acids micromol/dl (median/range)	12.3 (9.2-12.6)	5.7 (5.2-6.5)	13.6 (5.2-56.8)	NS
Trygliceride mg/dl (median/range)	98.5 (79-2691)	303 (55-428)	65 (28-82)	0.05
Cholesterol mg/dl (median/range)	90 (29-140)	85 (67-136)	92 (66-103)	NS

ALKP: alkaline phosphatase
ALT: alanine aminotransferase

- [A]:p value= 0.027 compared to control group
- [B]: p value= 0.009 compared to control group

Table 2. Summary of the descriptive data

The lipids were quantified and classified by type of sterols (animal: cholesterol, and plant: β-sitosterol, campesterol, and stigmasterol). Table 3 summarizes the total amount of sterols given to rats on parenteral nutrition. Table 4 summarizes the amount of lipids extracted from plasma and figure 1 shows their plasma concentrations. Animal and plant sterols were differently infused in the animals, with the latter generally accounting for a greater share than the former. Conversely, the same animal and plant sterols were inversely represented with cholesterol content largely predominant in the plasma of rats. Among phytosterols,

sitosterol had the highest plasma level in all the groups. Its highest concentrations were found in the ILG group, followed by the IL group. Controls had the lowest content, which, however, was not significant as differences among groups were not statistically significant. Plasma stigmasterol levels were significantly higher in both the IL and ILG groups. This time, differences were statistically significant (p=0.000). With respect to stigmasterol, a multiple pairwise comparison among groups confirmed a borderline statistically significant pairwise differences between IL and the control group (p:0.068). Plasma campesterol levels were similar across groups including the one receiving chow and differences were not significant.

Rat groups	Cholesterol (mg)	Campesterol (mg)	Stigmasterol (mg)	Sitosterol (mg)	Plant Sterol (mg)	Plant Sterol + Cholesterol (mg)
IL	3.0	0.68	0.72	2.5	3.9	6.8
ILG	2.72	0.60	0.64	2.9	3.5	6.2

Table 3. Overall amount of sterols parenterally injected into the rats

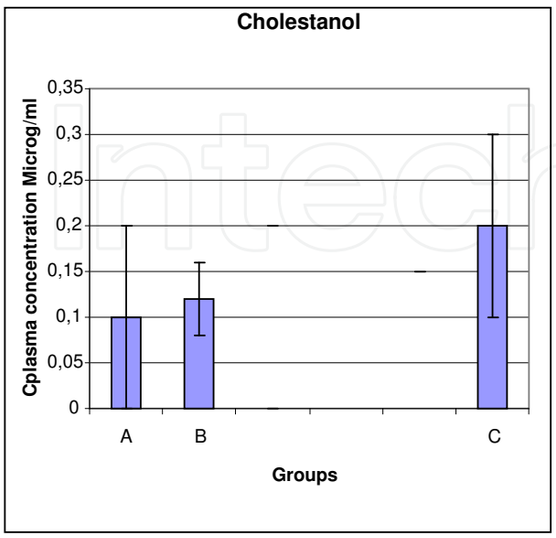
Rat group	Cholesterol mean (µg/ml)	Colestanol mean ±SD (µg/ml)	Campesterol mean ± SD (µg/ml)	Stigmasterol mean ± SD (µg/ml)	Sitosterol mean ± SD (µg/ml)	Total (µg/ml)
IL	>20.0	0.10 ± 0.1	0.9 ± 0.3	0.11 ± 0.01*	1.80 ± 0.3	22.91
ILG	>20.0	0.12 ± 0.04	0.7 ± 0.2	0.10 ± 0.1	1.50 ± 0.6	22.46
Chow	>20.0	0.20 ± 0.1	0.9 ± 0.2	0.014 ± 0.003	1.30 ± 0.5	22.41

*:p value= 0.068 when comparing IL with chow

Table 4. Quantification of plasma level of sterols in rats receiving parenteral, or oral nourishment

After regressing the amounts of provided sterols to the plasma levels identified, a strong positive association was found for the stigmasterol (R^2=0.83). Weaker associations were found for campesterol (R^2=0.37) and sitosterol (R^2=0.34).

None of the liver specimens showed abnormalities. Normal hepatic architecture and no evidence of PNAC or hepatic steatosis were found in all groups. Minor derangements were seen in scattered sections with signs of hepatic hypoxia around the central vein regions, which were likely due to the animal's sacrifice.



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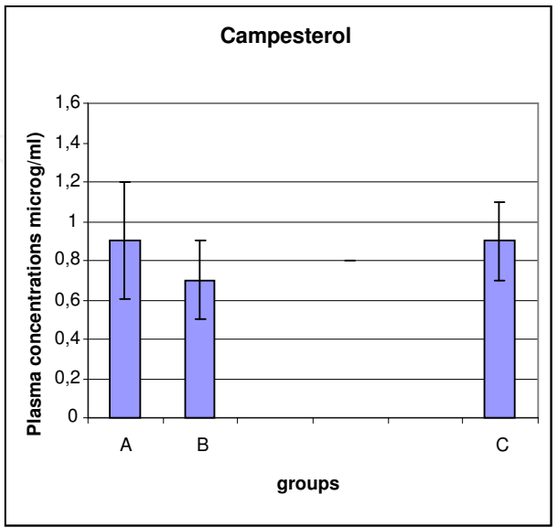

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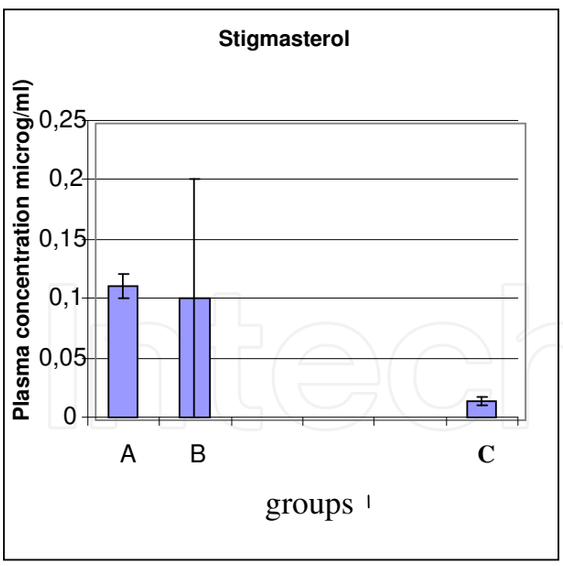

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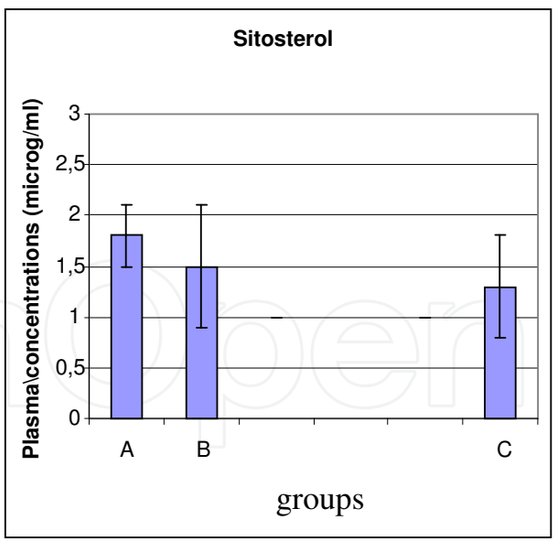

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Fig. 1. Plasma concentrations of sterols

4. Discussion

Scattered reports in the literature have linked PNAC to the vegetable sterol components of ILEs (1-5). Information on the sterol contents of ILEs, however, is currently scant and one of our previous purpose was to identify animal and plant sterols contained in the most representative ILEs. To this end, we selected ILE formulations as being representative in terms of their main oil component (soy, olive oil, fish oil variably combined with medium- or long-chain fatty acids, and purified triglycerides) and we quantified their animal and vegetal sterol contents (6). The relative weight of each sterol, however, varied significantly across formulations, but cholesterol and beta-sitosterol were the most represented followed by campesterol and stigmasterol. Our second hypothesis was then to verify the distribution of these sterols into plasma of rats and check their effects on liver structure. Therefore, in this study, we divided inbred weaning rats in three groups (two groups receiving TPN with or without glutamine and the third receiving chow) and we determined how sterols were distributed in plasma. Rats' liver status was also checked to verify whether sterols have changed histology in the short term.

While animal and vegetal sterols were differently represented across ILEs, with the latter generally present in greater amounts than the former, the opposite occurred in the plasma of rats regardless of their original nourishment. Cholesterol content was largely predominant in all groups, followed by sitosterol. Sitosterol was higher in those receiving TPN, although its level did not significantly differ among groups and did not correlate with the amount supplied. Campesterol was similarly represented in all three groups, while stigmasterol which had the lowest levels, was significantly correlated with the amount supplemented, and was even more significantly represented in the IL and ILG groups (p value 0.000). While expected in the TPN groups, it was a surprise to have found plant sterols considerably present in the plasma of rats fed with chow, given that sterols are usually not absorbed by the gastrointestinal system. As in humans, plasma concentrations of plant sterols, all detectable at low concentrations, vary at the individual level due to genetic polymorphism, it is possible that similar processes occur in animals. β-sitosterol, which is not endogenously produced, is rapidly secreted as neutral sterol into bile more than cholesterol and other plant sterols (9). This more rapid fractional turnover of β-sitosterol may indirectly indicate that the rigorous exclusion of sterols other than cholesterol in vertebrates may be essential for the maintenance of normal cholesterol homeostasis. When mutations affect the heterodimers transporter complex, sitosterolemia and hypercholesterolemia occur and atherosclerosis may develop. This process was highlighted in homologous animal proteins (10,11).

The association between phytosterols and PNAC has not been confirmed in this study carried out over a short term infusion of a high lipid load (40% vs 7 % of fat present in chow). These results are in contrast with those from other studies. La Scala et al, for instance, showed a direct correlation between the addition of lipids and the development of PNAC, which occurred in rats after 3 days of TPN infusion (5). On the contrary, Romestaing et al. proved that a long term highly saturated fat diet did not induce steatohepatitis in rats, but rather increased peripheral fat storage and thermogenesis (12). Interestingly, laboratory data obtained in our study, showed better parameters in the groups receiving ILEs' as compared to those in controls. Even though our original idea was to find an association between TPN and liver dysfunction, this finding would go to an opposite direction and be a sign of ILEs'

protection. The ILG group has the lowest GPT level as if the addition of glutamine had preserved hepatic functions. As previously reported in the literature, glutamine preserves liver glutathione after acute injuries and is able to cleanse the liver of the waste products (13). That is why we could have not found any histological alterations in the liver. The mechanisms through which phytosterols allegedly activate or, more precisely, influence or contribute to a cholestatic process in the liver are obscure. Perhaps the multifactorial origin of PNAC does not allow to separately assess the effects of each risk factor. Bypassing the gastrointestinal system, a high load of vegetal sterols with ILEs easily overload the blood stream and the liver. On top of the infused cholesterol, it is possible that both sterols act sinergically or competitively with the liver transporter system, which may displace either vegetal or animal sterol excretions and lead to bile duct plugging and initial inflammation. With continuous infusion of these sterols, infections, lack of oral intake, multiple drugs use, and surgery, the process might worsen. The finding that a patient developed a cirrhosis secondary to an autoimmune hepatitis and the combined occurrence of sitosterolemia due to a compound heterozygote ABCG8 gene mutation, may reflect a two-hit step and explain the need for liver transplantation, which completely reversed the phytosterol storage (14).

Stigmasterol was the only plant sterol which differentiated the three animal groups. A recent study investigating the role of different phytosterols (sitosterol, campesterol, and stigmasterol) in the induction of liver damage has shown that stigmasterol is a potent antagonist of the nuclear farnesoid X receptor FXR and the ligand-activated pregnane X receptor (a member also of the NR superfamily involved in the bile acid metabolism and inflammation), while sitosterol and campesterol had minimal or insignificant inhibitory effects in a human-derived liver cell line HepG2 (15). In addition to the agonist action of the liver X receptors (LXRs) (highly expressed in the liver, adipose tissue, intestine, kidney, and macrophages with involvement in sterol transport, fatty acid metabolism, glucose regulation, immunity , and cellular response), stigmasterol also interferes with the processing of sterol response element binding protein-2 (SREBP-2), which is a central regulatory factor in cholesterol metabolism. The nuclear receptor (NR) superfamily has been targeted as the main regulator in the adaptive response to cholestasis. In particular, the FXR NR1H4 seems to be the key regulator of intrahepatic bile acid levels. To maintain safe levels, it reprograms hepatic transcriptional factors reducing sinusoidal bile acid import, suppressing bile acid synthesis, and increasing intrahepatic bile acid efflux across canalicular and sinusoidal membranes. When FXR NR1H4 is lacking, hepatocytes are more susceptible to bile acid-induced injuries in rats. These rats, conversely, do not develop cholestasis if FXR NR1H4 is added back (16). Other studies have shown that phytosterols inhibit cholesterol 7-α-hydroxylase, which is the rate-limiting step for the conversion of cholesterol into bile acids (17). The mechanism by which stigmasterol disrupts cholesterol homeostasis activating LXR and suppressing the SREBP pathway was experimentally probed in a study in vitro where the authors also identified other sterols such as desmosterol, a cholesterol precursor (18). Apparently a double bond in the side chain of the sterol structure is responsible for the activation of LXR. Whether ILEs with high content of stigmasterol are crucial in the development of PNAC is yet to be proven. However, we believe that the solution's key lays in the interaction between phytosterols and phospholipids as proven in a model study in which the effect of the sterols on the molecular organization of the phospholipid monolayers was analyzed based on the compression modulus values (19). It was found that the incorporation of the phytosterols into the

phospholipid monolayers increased their condensation, without affecting the stoichiometry of the most stable phospholipids complexes. However, their stability was greatly affected. Cholesterol/phospholipids mixtures had the strongest interactions, while systems containing stigmasterol had the weakest.

The use of the intraperitoneal route to infuse TPN could be another explanation for the lack of hepatic derangements in this study. The intraperitoneal route is a well established procedure to infuse fluids and drugs in rodents (20). We decided to use the intraperitoneal route to avoid inserction of an central venous line, which would have exposed animals to two of the risk factors involved in the development of hepatic injuries: anesthesia and development of central venous line infections. Both factors would have greatly contributed to confuse the final outcome, due to the brief time of observation we planned. The intraperitoneal route was effective as the animals did not develop infections and were free to move without restrains.

One final thought goes to the selection of rats used in this study. To reduce the variability from selection bias, we decided to use inbred rats. In this way, differences could be totally attributed to the various feeding types as opposed to the inherited patterns of animals.

In conclusion phytosterols, which predominate in the infused ILEs compared to cholesterol, seem to be present in the plasma in quantities lower than cholesterol. No liver histological alterations occurred after a short-term high-fat parenteral infusions. These determinations will require further confirmation in long-term TPN supplementation and need to be analyzed taking into consideration the distribution of animal and plant sterols in the liver along with production of oxysterols.

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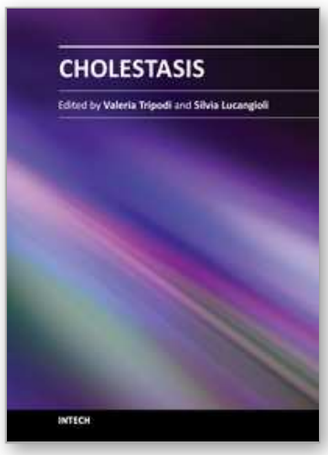

Cholestasis

Edited by Dr Valeria Tripodi

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This book covers different aspects on the understanding of mechanisms, effects, and management of cholestasis. This unique compendium contains important citations, an invaluable amount of research work, and many applications, which are outstanding resources for clinicians, pharmacists, biochemists, upper-level undergraduate, graduate, and continuing-education students who are dedicated to discovering new knowledge on cholestasis.

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