Cholestasis and biliary excretion of lipids induced by ethinylestradiol in rats fed polyunsaturated oils

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*Pharmacologic doses of ethinylestradiol are known to cause intrahepatic cholestasis in the rat. This is associated with a decrease in liver plasma membranes fluidity and a modification of the activity of membrane-bound enzymes related to bile flow. The intake of a diet containing highly polyunsaturated fats could modify the effects of estrogen, by increasing membranes fluidity due to their incorporation into structural phospholipids. To evaluate this action, rats were fed semipurified diets containing 17% corn (*Zea mais*) oil or rose hip (*Rosa moschata *Mill) seed oil during 20 days. Following this period, a group of animals were injected with ethinylestradiol during 5 days, while a control group and a pair fed group were included. Cholestasis was similar between corn and rose hip oils fed to ethinylestradiol-treated rats. The biliary excretion of cholesterol was higher in rose hip oil compared to corn oil-fed control rats. Biliary cholesterol/phospholipids ratio decreased in estrogen-treated rats compared to control animals. Our results show that the intake of highly polyunsaturated fatty acids did not modify the expected action of ethinylestradiol in rats.*

Keywords: rose hip seed oil; polyunsaturated fatty acids; ethinylestradiol; cholestasis; biliary lipids

Intoduction

The administration of pharmacologic doses of ethinylestradiol causes reversible cholestasis in rats.[1] This effect has been attributed to a reduction of liver plasma membranes fluidity,[2,3] which in turn affects the activities of membrane bound enzymes associated to bile flow. Some of the major determinants of membrane fluidity are the degree of saturation and length of the fatty acyl side chains of the structural phospholipids.[4] In a previous study,[5] we observed a modification of hepatocyte membranes fluidity after feeding rats a diet formula containing 20% highly polyunsaturated oils. In the current communication, we describe the effects of high doses of estrogen on biliary lipid composition and bile flow in rats fed semipurified diets containing rose hip and corn oils. These two fats differ in their polyunsaturated fatty acid (PUFA) content, and the polyunsaturation index (P/S) of rose hip oil more than doubles the corn oil respective value. A modified response to ethinylestradiol was expected, due to the incorporation of a high amount of PUFA into membrane lipids. However, most parameters showed similar values after both dietary regimens assayed, in spite of the differences in their fatty acid composition.

Methods and materials

Male Sprague-Dawley rats weighing 129 + 16.5 g (X ± SD) were housed individually in wire-bottomed cages, kept under controlled temperature and humidity conditions, and exposed to a 12-hour light/dark cycle. Animals were segregated into two groups of 24 rats each, fed semipurified diets containing rose hip seed oil (RHO) or corn oil (CO) as the only lipid source. The fatty acid composition of these oils in shown in *Table 1*. Both diets contained 17% vegetable oils, 20% casein, 4% mineral mix, 1% vitamin mix, 5% alphacel, 20% corn starch and 33% sucrose. Oils were kept in dark glass bottles in a cool chamber (4°C) Fresh diets were prepared weekly by adding the respective oils to the solid ingredients previously mixed.

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TABLE 1 Fatty acid composition of rose hip and corn oils (% methylesters)

Fatty acid	Corn oil	Rose hip oil
Myristic	–	0.1
Palmitic	9.0	3.7
Stearic	1.1	1.0
Oleic	28.9	17.6
Linoleic	59.5	49.8
Linolenic	1.5	27.8
$\frac{\text{Polyunsaturated}}{\text{Saturated}}$ index	6.0	16.2

Animals had free access to food and water during the first 20 days. On day 21, both experimental groups were distributed as follows: 1) Control, 2) Ethinylestradiol (EE), and 3) Pair-fed (PF) rats ($n = 8$). Control animals received propylene glycol, the vehicle for the drug, subcutaneously for 5 days. EE rats were administered 5 mg/kg body weight of 17-α-ethinylestradiol (in propylene glycol), subcutaneously for 5 days. Control and EE rats were fed ad libitum. PF rats were injected propylene glycol similarly to control animals, but were pair fed similarly to EE rats. On day 26, rats were anesthetized (sodium pentobarbital, 60 mg/kg), bile ducts were cannulated (PE-10 tubing), and bile samples were collected for 60 minutes, while body temperature and hydration were kept constant.

Bile samples were frozen until assayed (−20°C). Cholesterol was quantitated by enzymatic methods.[6] Bile salts were measured by the method of Talalay,[7] modified by Turley et al.[8] Phospholipdis were assayed by quantitating phosphorus.[9]

Results are expressed as mean values ± standard deviation. Differences were estimated by Student *t* test for independent samples at a significance level of $P < 0.05$.

Results

Rats were fed the experimental diets for 20 days before ethinylestradiol administration in order to promote the incorporation of the dietary fatty acids into their body tissues, which could modify the effects of estrogen.

Table 2 shows the food intake and body growth of rats fed CO and RHO throughout the experience. From day 1 to 20, both parameters exhibited similar patterns. Animals showed comparative acceptability for both diets, and significant differences became evident during the pharmacological treatment and dietary restriction periods. As was expected, the administration of large doses of estrogen caused a sharp reduction in food intake of EE rats ($P < 0.001$), which paralleled body weight loss during this period ($P < 0.01$) due to the anorectic effect of the drug. PF groups, in spite of a food intake similar to EE rats, showed a greater body weight loss compared to Control groups ($P < 0.001$).

Ethinylestradiol is known to induce liver growth due to hepatocyte hyperplasia.[10] Our results show that relative liver weight of EE rats was increased ($P < 0.001$), and only PF group fed RHO showed a reduced value ($P < 0.001$).

The effect of ethinylestradiol administration on bile flow and lipid concentration is shown in *Table 3*. Large doses of estrogen led to a marked reduction in bile flow (54.2% vs. Control rats fed CO [$P < 0.01$] and 64.3% versus Control animals fed RHO [$P < 0.001$], respectively.) This parameter was not affected by dietary restriction per se. An increase in biliary lipid concentration due to EE induced cholestasis was expected.[11] An increase in the amount of cholesterol of 120% was observed in EE rats fed CO compared to their respective Controls ($P < 0.001$). A similar result was observed in EE rats fed RHO, with an increment of 105% versus its Control ($P < 0.001$). Augmented concentrations of phospholipids were also evident in both EE groups: Values were about 290% greater versus the respective CO and RHO fed Controls ($P < 0.001$). The rise in bile acids concentration was more important in EE rats after the intake of RHO, showing an increment of about 140% versus Control ($P < 0.001$), while it reached about 68% in EE rats fed CO compared to their respective Control group ($P < 0.01$).

The biliary lipid concentration measured in PF animals showed different responses: The amount of phospholipids was increased in PF rats fed both vegetable oils ($P < 0.001$), while cholesterol concentration was reduced in animals fed CO ($P < 0.01$) and, on the contrary, was increased in rats fed RHO ($P < 0.01$). Bile acids concentration was significantly higher in an-

Table 2 Food intake, body weight change, and relative liver weight of rats before and during ethinylestradiol administration

	Corn oil diet			Rose hip oil diet		
	Control $n = 6$	EE $n = 7$	Pair-fed $n = 7$	Control $n = 8$	EE $n = 8$	Pair-fed $n = 8$
Food intake (g)						
Days 1–20	17.3 ± 1.4	16.3 ± 2.7	17.7 ± 1.7	15.8 ± 1.8	17.1 ± 1.5	17.1 ± 1.6
Days 21–25	16.2 ± 3.0	8.5 ± 2.6[c]	6.9 ± 1.8[c]	14.0 ± 2.3	7.6 ± 1.2[c]	7.5 ± 1.4[c]
Body weight change (g)						
Days 1–20	155.3 ± 10.1	149.0 ± 23.0	146.3 ± 27.8	132.0 ± 19.7	150.1 ± 18.2	143.0 ± 22.7
Days 21–25	37.2 ± 11.8	−12.0 ± 6.3[c]	−21.9 ± 7.4[c]	23.3 ± 4.5	−13.8 ± 4.3[c]	−19.1 ± 6.1[c]
Relative liver weight (%)	3.2 ± 0.2	5.6 ± 0.8[c]	3.1 ± 0.3	4.0 ± 0.5	5.6 ± 0.4[c]	3.0 ± 0.4[c]

Values represent mean ± SD. Significant difference from Control value c: $P < 0.001$.

Table 3 Bile flow and lipid concentration in rats after treatment

	Corn oil diet			Rose hip oil diet		
	Control $n = 5$	EE $n = 6$	Pair-fed $n = 7$	Control $n = 7$	EE $n = 7$	Pair-fed $n = 7$
Bile flow (μL/g liver/min)	0.96 ± 0.39	0.44 ± 0.17[b]	1.20 ± 0.17	1.27 ± 0.24	0.45 ± 0.13[c]	1.35 ± 0.32
Biliary concentration (μmol/mL)						
Cholesterol	0.55 ± 0.18	1.21 ± 0.20[c]	0.33 ± 0.10[b]	0.61 ± 0.08	1.25 ± 0.23[c]	0.81 ± 0.17[a]
Phospholipids	2.95 ± 1.08	11.39 ± 2.88[c]	5.59 ± 0.47[c]	3.83 ± 1.34	14.97 ± 3.73[c]	7.31 ± 1.72[c]
Bile acids	27.48 ± 12.21	46.05 ± 9.08[b]	25.60 ± 5.00	19.87 ± 5.50	47.89 ± 9.38[c]	31.71 ± 4.46[a]
Cholesterol / Phospholipids	0.20 ± 0.10	0.11 ± 0.04	0.06 ± 0.02[a]	0.18 ± 0.07	0.07 ± 0.03[a]	0.12 ± 0.03[b]

Values represent mean ± SD. Significant difference from Control value a: $P < 0.05$, b: $P < 0.01$, c' $P < 0.001$.

Table 4 Biliary lipid secretion in rats after treatment

	Corn oil diet			Rose hip oil diet		
	Control $n = 5$	EE $n = 6$	Pair-fed $n = 7$	Control $n = 7$	EE $n = 7$	Pair-fed $n = 7$
Biliary secretion (nmol/g liver/min)						
Cholesterol	0.52 ± 0.22	0.55 ± 0.27	0.39 ± 0.14	0.77 ± 0.15	0.54 ± 0.11[b]	1.06 ± 0.20[b]
Phospholipids	3.72 ± 1.63	4.65 ± 0.99	6.64 ± 0.70[c]	4.80 ± 1.81	5.97 ± 0.72	9.90 ± 2.86[b]
Bile acids	25.25 ± 3.97	19.17 ± 4.59[a]	30.97 ± 9.54	24.62 ± 5.96	20.74 ± 4.50	43.09 ± 10.60[b]

Values represent mean ± SD. Significant difference from Control value a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$.

imals fed RHO ($P < 0.05$), and showed no changes in those fed CO.

As a consequence of the steep increase in biliary phospholipids concentration in EE rats, compared to changes observed in those of cholesterol, C/PL ratio was lower in these animals versus their Controls. This decrease was significant after RHO feeding only ($P < 0.05$), where it was about 40% the respective value of C/PL ratio in Control rats fed this highly polyunsaturated fat.

Biliary secretion rate of lipids, obtained from bile flow and lipid concentration data, are presented in *Table 4*. Control rats fed RHO exhibited a higher secretion rate of cholesterol than those fed CO ($P < 0.05$). This increased cholesterol output is due to the higher bile flow and biliary cholesterol concentration values observed in these animals compared to Control rats fed CO. Ethinylestradiol treatment caused a significant lowering of bile cholesterol output in rats fed RHO ($P < 0.01$), while this effect was not observed in rats fed CO. Biliary secretion rates of phospholipids were similar in EE groups fed both experimental diets.

On the other hand, bile acids output was reduced in EE groups, due to a well-known effect of this estrogen,[11] although the difference versus Control was significant after CO feeding only ($P < 0.05$). Pair-fed rats fed CO showed an increased bile output of phospholipids ($P < 0.001$), while those fed RHO exhibited an outstanding increase in biliary output of cholesterol, phospholipids, and bile acids, compared to their Controls ($P < 0.01$).

Discussion

Vegetable oils used in this study contain a high proportion of linoleic acid (18:2 n-6). CO content of this PUFA is 59.5%, while RHO value is 49.8%. They differ mainly in the linolenic acid (18:3 n-3) content: RHO is particularly rich in this fatty acid, and its content was over 18 times higher than the CO amount. RHO is not considered an edible fat, and at present it is used in cosmetics. In this experience, RHO was used as a means of promoting the incorporation of a high amount of PUFA into the body tissues of rats, while CO was employed for comparison. The experimental design included groups of rats pair fed to EE animals to separate the effects of estrogen from those of decreased food intake. Our results indicate that the effects of pharmacological doses of ethinylestradiol on bile flow and biliary lipids in rats were not modified by a high intake of dietary PUFA.

The cholestatic effect of estrogen is attributed to induced changes in the lipid composition of liver plasma membranes. In the treated rats, an increased C/PL ratio has been described, associated to hepatocyte membranes fluidity decrease.[12,13] Estrogen impairs the capacity of the liver to secrete bile acids independent bile fraction and noncompetitively inhibit Na,K-ATPase activity in liver plasma membranes, among other actions.[14,15] This enzyme is located mainly in the basolateral and sinusoidal domains of the hepatocyte, and is very sensitive to the lipid microenvironment.[16,17] Many authors have described a di-

rect relationship between the reduced activity of Na,K-ATPase bound to membranes and a decreased bile flow.[2] However, current evidence demonstrates that it is not possible to attribute the cholestatic effect of estrogen to this action only.[18]

Our data demonstrate that the intake of high quantities of PUFA did not alter the cholestatic effect of ethinylestradiol. No significant differences were observed after both dietary regimens assayed in EE rats, in spite of the higher P/S ratio of RHO. Food intake did not affect bile flow, as Control and PF rats showed similar values. This parameter is also dependent on a series of variables, including the hydration level of the animals, anesthetic agent used, body temperature, and fasting, all of which were under control.

The biliary concentration of all lipid classes was increased after estrogen therapy. This is an expected result related to the ability of the drug to inhibit the hepatic synthesis of cholesterol and bile acids[19,14] and to increase the intracellular cholesterol esterification,[2] while it is excreted in bile. The simultaneous increase in the amount of phospholipids is in agreement with previous reports.[20] The relative changes in the concentration of these two lipid species caused the biliary C/PL ratio to be lower in EE compared to Control values. Dietary restriction also caused a decrease in C/PL ratio, probably as a consequence of the altered metabolism of lipids due to the lack of nutrients. An interesting difference was observed in PF rats fed either vegetable oil: Animals fed RHO exhibited increased bile concentration and secretion of cholesterol, compared to the Control respective group, while CO fed rats showed the opposite. Since the main differences between these two fat sources is their linolenic acid content, we attribute the observed effects to the degree of polyunsaturation of CO and RHO. The difference in the relative fatty acid composition of dietary oils may have thus influenced bile composition, reflecting a change in the hepatic cholesterol metabolism.

Similar biliary output of lipids was observed in EE rats, compared to non-treated Control animals, due to the combined effects of the increase in biliary lipids concentration and the cholestatic action of estrogen per se. Two exceptions to this general principle were observed: the lower biliary output of cholesterol in EE rats fed RHO and lower bile acids secretion in those fed CO. The former should be related to the increased biliary output of cholesterol in Control rats fed RHO. The unexpected rise in lipids secretion measured in PF rats fed this highly polyunsaturated fat could be related to an impaired metabolism of lipids that induced the removal of these compounds from the liver.

The lowering in bile acids secretion rate observed after pharmacological treatment is related to ethinylesteradiol inhibition of microsomal 7-α-hydroxylase activity, thus decreasing bile acids synthesis in the liver.[21] Accordingly, low bile acids secretion rates in EE rats could reduce bile acids dependent bile fraction, which correlates well with the impaired bile flow observed in these animals. Moreover, decreased Na,K-ATPase activity in liver plasma membranes is known to modify bile acids uptake at the sinusoidal domain of the hepatocyte,[12] which may also have contributed to our results. Most of the differences observed between PF rats fed CO and RHO should be attributed to the effects of the lipid source of the experimental diets, since most other variables were carefully controlled. This allows us to conclude that the lipid quality of the diet exerted important effects on biliary lipid composition. However, our results demonstrate that the effects of high doses of ethinylestradiol on bile flow and composition were not modified by the intake of dietary PUFA. Therefore, the effects of the inclusion of these fatty acids in liver plasma membranes did not counteract the expected action of estrogen.

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