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Oxidative Stress during Development of Alcoholic Fatty Liver: Therapeutic Potential of Cacao Polyphenol

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The lipid and antioxidative/oxidative profiles of livers from rats fed an ethanol liquid diet for 8 weeks provided evidence for an involvement of oxidative stress (*e.g.*, phospholipid peroxidation) in the development of alcoholic fatty liver (AFL), possibly in an early stage. Cacao polyphenol supplementation produced an ameliorating effect, and may help in AFL prevention.

Key words: alcoholic fatty liver; lipid peroxide; tocopherol; cacao polyphenol; triglyceride

Most heavy drinkers develop alcoholic fatty liver (AFL),[1)] which is a major risk factor for advanced liver diseases such as steatohepatitis and fibrosis. An understanding of the effects of ethanol on the liver could lead to therapeutic treatments to reverse AFL, and help in the prevention of advanced liver injuries. Because lipid peroxide accumulation is often found in the hepatic tissues of alcoholic patients with steatohepatitis and fibrosis,[2)] it has been hypothesized that oxidative stress plays an important role in the development of AFL. Consistent with this hypothesis, Song *et al.*[3)] reported that rats fed an ethanol liquid diet developed AFL and showed high levels of thiobarbituric acid reactive substances (TBARS), which are secondary lipid oxidation products in the liver.

However, there are conflicting data that chronic ethanol administration does not lead to significant changes in TBARS levels or antioxidant enzyme activities in rats.[4)] Therefore, the role of oxidative stress in AFL development remains unclear. One reason for this uncertainty could be the potential methodological problems, such as selectivity and sensitivity,[5)] of the TBARS assay used in these studies.[3,4)]

In order to evaluate whether oxidative stress (lipid peroxidation) actually occurs during AFL development, rats were fed an ethanol liquid diet to create an AFL model.[6)] Liver homogenates from these rats were used to measure phospholipid hydroperoxide (PLOOH) using chemiluminescence detection and high-performance liquid chromatography (CL-HPLC).[7)] PLOOH is a primary product of phospholipid oxidation. Thus, an increase in PLOOH is a direct reflection of *in vivo* oxidative stress.[7)] In addition, the effect of dietary antioxidant supplementation on AFL development was evaluated using cacao polyphenol (which mainly consists of epicatechin and its polymer).[8)]

Six-week-old male Wistar rats were obtained from Japan SLC (Tokyo, Japan), and housed individually in a temperature- (23 °C) and humidity-controlled room with a 12-h light:dark cycle. After acclimatization for 1 week, the rats were divided into four groups of 10 rats each. The rats were pair-fed on the ethanol or non-ethanol (isocaloric carbohydrate) liquid diet,[6)] with or without cacao polyphenol (1.4 g/L; Barry Callebaut Ltd., Zurich, Switzerland) (Table 1). The four groups were classified as follows: non-ethanol + control group, non-ethanol + cacao group, ethanol + control group, and ethanol + cacao group. Body weights were measured twice a week during a feeding period of up to 8 weeks. After feeding for 4 weeks, three rats from each group were starved for 16 h, and anesthetized with isoflurane before their livers were removed and perfused. Livers were collected from the remaining rats in each group ($n = 7$) at the end of the 8-week feeding period. This study was approved by the Institutional Animal Care and Use Committee (Permission number: SKL-J-11016) and carried out according to the Sankyo Labo Service Corporation, INC Animal Experimentation Regulations (Ibaraki, Japan).

Liver homogenates (20% w/v) were prepared with an aqueous solution containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.9% NaCl. Total lipids were extracted from the homogenates using the Folch method.[9)] Total lipid extracts were subjected to triglyceride (TG) analysis using a commercial kit (Wako, Osaka, Japan) as well as PLOOH (*i.e.*, phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH)) determination using CL-HPLC. CL-HPLC analysis was conducted using a Finepak SIL NH2-5 column (4.6 × 250 mm; Japan Spectroscopic Co., Tokyo, Japan). The eluent was 2-propanol/methanol/water (135:45:20, v/v/v), and the flow rate was 1 mL/min. A CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan) was used for post-column CL detection. A mixture of luminol and

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Abbreviations: AFL, alcoholic fatty liver; TBARS, thiobarbituric acid reactive substances; PLOOH, phospholipid hydroperoxide; CL-HPLC, chemiluminescence detection and high-performance liquid chromatography; TG, triglyceride; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide

cytochrome *c* in 50 mM borate buffer (pH 10.0) was used as a hydroperoxide-specific post-column CL reagent, with flow rate of 2 mL/min. Calibration was performed using standard PLOOH.[10] In addition to TG and PLOOH analysis, retinol and α-tocopherol were extracted from the livers, and measured using HPLC.[11]

The data were expressed as the mean ± SD. Two-way analysis of variance was performed using Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences were considered significant at $p < 0.05$.

A liquid ethanol diet can reportedly induce AFL by pair-feeding without special techniques or equipment.[3] After 4 or 8 weeks of feeding, the average body weights (initial and final) in each group were not significantly different (non-ethanol + control group, non-ethanol + cacao group, ethanol + control group, and ethanol + cacao group) (Table 2). Also, daily caloric intake did not differ among the groups. Therefore, in this study, experimental diets were considered to be properly isocalorically pair-fed to rats. The following results and discussion addresses whether lipid peroxidation actually occurs during AFL development by comparing the non-ethanol + control and ethanol + control groups, followed by the effects of cacao polyphenol on oxidative stress and AFL.

Table 1. Composition of the Ethanol and Non-Ethanol Liquid Diets Supplemented with or without Cacao Polyphenol

	Non-ethanol		Ethanol	
	Control	Cacao	Control	Cacao
Caloric ingredients (energy %)				
Protein	17.0	17.0	17.2	17.2
Carbohydrates	47.0	47.0	10.5	10.5
Fat	36.0	36.0	36.5	36.5
Ethanol	—	—	35.8	35.8
Total	100.0	100.0	100.0	100.0
Non-caloric ingredients (g/L)				
Cacao polyphenol[a]	—	1.4	—	1.4
Vitamin mix[b]	2.5	2.5	2.5	2.5
Mineral mix[b]	8.8	8.8	8.8	8.8

[a]The total polyphenol content was determinated according to the Folin-Ciocalteu colorimetric method.[16]
[b]See reference 6.

Based on the results from the non-ethanol + control and ethanol + control groups, a liquid ethanol diet can induce TG accumulation in the liver. TG levels increased to 25.9 mg/g liver after 8 weeks of ethanol administration (Table 2). As suggested by a previous report,[12] ethanol could have impaired mitochondrial and/or peroxisomal fatty acid oxidation, causing liver TG accumulation. The liver TG concentration of 25.9 mg/g could be interpreted as the initial stage of AFL development, which would be useful in elucidating the primary contribution of ethanol to liver oxidative stress (lipid peroxidation) during AFL development.

Retinol levels lend more support for early stage AFL in the rats following the 8-week ethanol diet. Lower hepatic retinol levels appear early in alcoholics,[13] and in this study, the level of liver retinol was low in the ethanol + control group, as compared to the non-ethanol + control group (Table 2). The mechanism underlying the decrease may be due to ethanol induction of cytochrome P450 enzymes in the liver, thus enhancing catabolism of retinol.[14]

Once AFL development was confirmed, oxidative stress was evaluated by analyzing liver α-tocopherol, a major antioxidant molecule. Since it is known that hepatic α-tocopherol levels change in proportion to TG levels,[15] α-tocopherol concentration was calculated against TG (Fig. 1). A comparison of the non-ethanol + control and ethanol + control groups revealed that liver α-tocopherol was significantly reduced after 8 weeks of ethanol intake. Since such a decrease was not observed after 4 weeks of the ethanol diet, it is possible that oxidative stress occurred in the liver of rats receiving ethanol during weeks 4–8. To further evaluate this possibility, liver PLOOH was determined by CL-HPLC. In a CL-HPLC chromatogram of liver total lipids, PCOOH and PEOOH were identified as the predominant PLOOH forms (data not shown). After 8 weeks of ethanol administration, the ethanol + control group showed high levels of liver PCOOH and PEOOH significantly, as compared with the non-ethanol + control group (Fig. 1). Based on these findings (Table 2

Table 2. Body Weight, Food Intake, and Liver Parameters of Rats Fed with the Ethanol or Non-Ethanol Liquid Diet Supplemented with or without Cacao Polyphenol for 4 and 8 Weeks

	4 weeks (n = 3)				8 weeks (n = 7)			
	Non-ethanol		Ethanol		Non-ethanol		Ethanol	
	Control	Cacao	Control	Cacao	Control	Cacao	Control	Cacao
Initial weight (g)	173.2 ± 10.7	174.0 ± 9.4	174.8 ± 7.2	176.0 ± 6.7	169.8 ± 6.5	170.1 ± 6.3	170.1 ± 6.5	169.9 ± 6.2
Final weight (g)	206.1 ± 15.4	205.6 ± 4.3	207.8 ± 11.9	212.5 ± 24.2	270.0 ± 6.1	279.4 ± 17.4	278.5 ± 18.8	280.9 ± 4.0
Carorie intake (kcal/kg weight)	208.7 ± 6.7	208.1 ± 7.4	199.5 ± 3.3	195.2 ± 6.9	208.4 ± 8.7	212.3 ± 10.6	203.2 ± 9.3	209.6 ± 6.3
Ethanol intake (g/kg weight)	—	—	10.0 ± 0.2	9.8 ± 0.3	—	—	10.2 ± 0.5	10.5 ± 0.3
Cacao polyphenol intake (mg/kg weight)	—	285.4 ± 10.1	—	267.8 ± 9.4	—	291.3 ± 14.5	—	287.5 ± 8.7
TG (mg/g of liver)	10.1 ± 2.5	7.4 ± 1.5	18.7 ± 4.2#	23.8 ± 1.1*#	13.9 ± 4.6	12.9 ± 4.6	25.9 ± 3.9#	14.7 ± 2.8*
Retinol (μg/g of liver)	355.4 ± 33.8	355.8 ± 47.1	154.1 ± 47.6#	101.8 ± 23.0#	100.2 ± 33.4	133.3 ± 22.9*	38.8 ± 9.3#	52.1 ± 12.2#

Data points represent the mean ± SD. *Significantly different from the corresponding control value ($p < 0.05$); #significantly different from the corresponding non-ethanol group ($p < 0.05$).

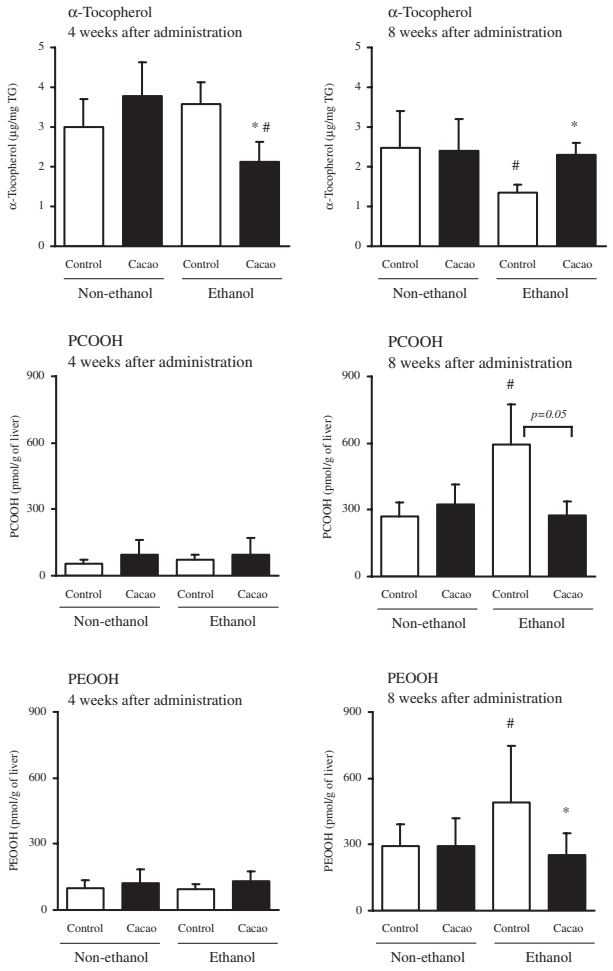

Fig. 1. Levels of α-Tocopherol and PLOOH (PCOOH and PEOOH) in the Liver of Rats Fed a Liquid Ethanol Diet with and without Cacao Polyphenol.

Detailed conditions are described in the text. Data points represent the mean ± SD. *Significantly different from the corresponding control value ($p < 0.05$); #significantly different from the corresponding non-ethanol group ($p < 0.05$).

and Fig. 1), it is likely that ethanol gradually induces TG accumulation in the liver at 4 weeks after initiation of the ethanol diet. Such an accumulation of liver TG would cause oxidative stress. This is one possible reason for the decreased levels of α-tocopherol and increased concentrations of PLOOH in the livers of rats receiving the liquid ethanol diet for 8 weeks.

The effect of the antioxidant cacao polyphenol on typical AFL symptoms was also examined. In both the ethanol + control and ethanol + cacao groups, higher TG concentrations were found in livers at 4 weeks after initiation of each liquid ethanol diet regimen. However, at 8 weeks the liver TG level of the ethanol + cacao group was significantly lower than that of the ethanol + control group (Table 2), suggesting that cacao polyphenol can prevent ethanol-induced TG accumulation in the liver. To the best of our knowledge, this is the first report implicating a protective effect of cacao polyphenol against TG accumulation during AFL development. Although cacao polyphenol did not ameliorate ethanol-induced retinoid catabolism, supplementation of cacao for 8 weeks could prevent an ethanol-induced decrease in α-tocopherol (Fig. 1). In addition, at week 8, cacao significantly reduced the ethanol-induced increase in PEOOH, and showed a tendency in PCOOH ($p = 0.05$), as compared with the ethanol + control group. These findings suggest that during AFL development, TG accumulation may induce oxidative stress in the liver, and that this stress can be effectively reduced by cacao polyphenol. The decrease could be achieved directly through antioxidant activity and/or indirectly by lowering TG levels. Further studies are needed to evaluate these possibilities.

In conclusion, in this study, analyses of the TG profile, antioxidant status (α-tocopherol), and oxidative stress marker (PLOOH) in the livers of rats fed an ethanol liquid diet provided evidence that oxidative stress (lipid peroxidation) occurs during an early stage of AFL development. As a preventive strategy, cacao polyphenol might help avert AFL development by reducing TG accumulation and oxidative stress. Further studies on ethanol action, as well as the effects of cacao polyphenol on AFL, are needed to provide therapeutics for the prevention of AFL and advanced liver injuries.

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