Chronic treatment with bark infusion from *Croton cajucara* lowers plasma triglyceride levels in genetic hyperlipidemic mice

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Abstract: Aqueous infusion and preparations containing dehydrocrotonin (DHC) and essential oil from *Croton cajucara* bark were tested for plasma lipid–lowering effects in genetically modified hyperlipidemic mice. Two mouse models were tested: 1) primary hypercholesterolemia resulting from the LDL–receptor gene knockout, and 2) combined hyperlipidemia resulting from crosses of LDL–receptor knockout mice with transgenic mice overexpressing apolipoprotein (apo) CIII and cholesteryl ester–transfer protein. Mice treated with bark infusion, DHC, essential oil, or placebos for 25 days showed no signals of toxicity as judged by biochemical tests for liver and kidney functions. The bark infusion reduced triglyceride plasma levels by 40%, while essential oil and DHC had no significant effects on plasma lipid levels. The bark infusion treatment promoted a redistribution of cholesterol among the lipoprotein fractions in combined hyperlipidemic mice. There was a marked reduction in the VLDL fraction and an increase in the HDL fraction, in such a way that the (VLDL + LDL)/HDL ratio was reduced by half. The bark infusion treatment did not modify cholesterol distribution in hypercholesterolemic mice. In conclusion, *C. cajucara* bark infusion reduced plasma triglycerides levels and promoted a redistribution of cholesterol among lipoproteins in genetically combined hyperlipidemic mice. These changes modify risk factors for the development of atherosclerotic diseases.

Key words: hyperlipidemia, transgenic mice, *Croton cajucara*, dehydrocrotonin, cholesterol.

Résumé : On a examiné une perfusion aqueuse et des préparations contenant de la déhydrocrotonine (DHC) et de l'huile essentielle provenant de l'écorce de *Croton cajucara* pour déterminer les effets réducteurs des lipides plasmatiques chez des souris hyperlipidémiques génétiquement modifiées. Deux modèles de souris ont été utilisés : 1) hypercholestérolémie primaire provoquée par l'inactivation du gène du récepteur des LDL et 2) hyperlipidémie combinée provenant de croisements de souris ayant subi une inactivation du récepteur des LDL et de souris transgéniques surexprimant l'apolipoprotéine CIII et la protéine de transfert de l'ester de cholestérol. Les souris traitées avec la perfusion d'écorce, la DHC, l'huile essentielle ou des placebos pendant 25 jours n'ont montré aucun signe de toxicité comme l'ont indiqué des tests biochimiques sur les fonctions hépatiques et rénales. La perfusion d'écorce a réduit les taux de triglycérides plasmatiques de 40 % alors que l'huile essentielle et la DHC n'ont pas eu d'effet significatif sur les taux de lipides plasmatiques. Le traitement par perfusion d'écorce a favorisé une redistribution du cholestérol entre les fractions de lipoprotéines chez les souris souffrant d'hyperlipidémie combinée. La fraction de VLDL a diminué de manière significative et la fraction de HDL a augmenté de sorte que le rapport (VLDL+LDL)/HDL a été réduit de moitié. Le traitement par perfusion d'écorce n'a pas modifié la distribution de cholestérol chez les souris hypercholestérolémiques. En conclusion, la perfusion d'écorce de *C. cajucara* a réduit les taux de triglycérides plasmatiques et favorisé une redistribution de cholestérol entre les lipoprotéines chez les souris atteintes d'hyperlipidémie combinée d'origine génétique. Ces variations modifient les facteurs de risque de l'apparition des maladies athérosclérotiques.

Mots clés : hyperlipidémie, souris transgéniques, *Croton cajucara*, déhydrocrotonine, cholestérol.

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Introduction

Disturbances in plasma lipid and lipoprotein metabolism may lead to an increased risk of atherosclerosis. High levels of low-density lipoprotein (LDL)-cholesterol and low levels of high-density lipoprotein (HDL)-cholesterol are well established risk factors for coronary heart disease (Goldstein and Brown 1977; Gordon and Rifking 1989; Goldstein and

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Brown 2001). The ability of several plant-derived natural products to control hyperlipidemia and associated diseases has been tested (Wang and Ng 1999; Craig 1999; Urizar et al. 2002). *Croton cajucara* Benth. (Euphorbiaceae), a plant grown mainly in the Amazonian area, has been used in Brazil to treat diabetes, hypertension, and high blood cholesterol levels. Its leaves and bark are popularly used, either in the form of infusions or dry stem-bark pills (Maciel et al. 2000). A major constituent extracted from the bark is the clerodane diterpene trans-dehydrocrotonin (DHC) (Maciel et al. 1998). Previous studies have demonstrated the hypolipidemic effects of *C. cajucara* alcoholic extract in rats (Farias et al. 1996) and DHC treatment in mice (Silva et al. 2001*a*), when both species were fed a cholate-containing high-fat, high-cholesterol diet. DHC treatment has been shown to suppress the transient increase in plasma cholesterol and triglycerides induced by Triton WR1339 in mice (Silva et al. 2001*b*) and ethanol-induced hypertriglyceridemia in rats (Silva et al. 2001*c*). The essential oil fraction isolated from the plant hexane/chloroform extracts contains steroids such as beta-sitosterol, and flavonoids such as kaempferol (Maciel et al. 2000). The lipid-lowering potential of the *C. cajucara* essential oil has never been evaluated.

Transgenic animals over- or underexpressing target genes involved in lipoprotein metabolism have been useful models with which to investigate factors and therapies affecting lipoprotein metabolism and the development of atherosclerosis (Asset et al. 1999; Breslow 1996; Paigen et al. 1994). In this respect, LDL-receptor knockout mice (Ishibashi et al. 1993) present the phenotype of human primary hypercholesterolemia, exhibiting high levels of LDL-cholesterol. The progeny from crosses of LDL-receptor knockout mice and transgenic mice overexpressing apoCIII and cholesteryl ester transfer protein (CETP) display the features of the familial combined hyperlipidemia (Masucci-Magoulas et al. 1997), a common poligenic inherited lipid disorder that affects 1% to 2% of Western populations. These mice have hypercholesterolemia and hypertriglyceridemia as a consequence of high levels of very low-density lipoprotein (VLDL) and LDL, and low levels of HDL.

In this study, we investigated the hypolipidemic potential of aqueous infusion and plant preparations containing either DHC or essential oil from *C. cajucara* bark in these two genetically modified mouse models.

Materials and methods

Preparations of *C. cajucara* bark fractions

The bark of *C. cajucara* was collected from an experimental plantation in Benfica, near Belém, Pará, Brazil. A voucher specimen (No. 247) was identified by Nelson A. Rosa and is deposited in the IAN Herbarium, Belém, Brazil. *C. cajucara* bark was air dried for seven days, finely milled, and stored in a dark glass flask inside a dissector at room temperature. The aqueous infusion was freshly prepared every day. Air-dried milled bark was suspended in boiling water to a final concentration of 5% (w/v). The infusion stood at room temperature for 15 min before being filtered with cheese cloth. This procedure was chosen because it closely follows the infusion regularly prepared and drunk by general users. Essential oil was prepared, as described elsewhere (Hiruma-Lima et al. 2002), using steam to distill air-dried milled bark in benzene for 6 h. Preliminary gas-chromatography/mass spectrometry analysis, confirmed by ^{13}C-NMR spectra, showed that the main components of this fraction were $C_{15}H_{24}$ sesquiterpenes, α-copaene (20.9%), and cyperene (29%); there was no DHC. Essential oil was emulsified in 12% Tween 80 before being administered to the animals. DHC was obtained from *C. cajucara* barks in accordance with the method described by Souza-Brito et al. (1998). Briefly, air-dried stalk-bark was extracted using a Soxhlet apparatus, and the crude crystals formed in a concentrated hexane solution were recovered after a few days. A pure compound was obtained after repeated crystallizations in isopropanol. The DHC was emulsified in 12% Tween 80 before being administered to the animals.

Animals and treatments

Animal protocols were approved by the university's Ethics Committee and conform with *The Guidelines on the Handling and Training of Laboratory Animals*, published by the Universities Federation for Animal Welfare, UK (1992). LDL-receptor knockout mice ($LDLr^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, Me.). ApoCIII (line 3707) and cholesteryl ester transfer protein (natural flanking region (NFR)-CETP, line 5203) transgenic founders were provided by Dr. A.R. Tall (Columbia University, N.Y.), and have been described elsewhere (Walsh et al. 1993; Jiang et al. 1992). Animals were bred and maintained in a temperature-controlled room (22 ± 2 °C) with 12 h light : 12 h dark cycle, where they had free access to water and food (rodent chow, Nuvital CR1, Curitiba, PR, Brazil). ApoCIII and CETP transgenic mice were bred, and the resulting CIII/CETP double transgenics were crossbred with $LDLr^{-/-}$ mice to produce CIII/CETP/$LDLr^{+/-}$ mice. $LDLr^{-/-}$ mice were screened using PCR (Ishibashi et al. 1993), apoCIII was determined using the fasting plasma level of triglycerides (TG > 300 mg/dL), and CETP was determined using the plasma CETP activity assay described below. Mice received one of the following treatments, once a day through a gastric tube, for 25 consecutive days: 5% bark infusion (500 mg/kg body weight); water (10 mL/kg body weight); DHC (100 mg/kg body weight); essential oil (100 mg/kg body weight); or 12% Tween 80 (10 mL/kg body weight). The amount of bark infusion given daily to the mice was equivalent to the 2.5 cups drunk by an adult human.

Plasma biochemical analysis

Plasma was isolated from blood collected from the retroorbital plexus of anesthetized mice with heparinized hematocrit tubes. Levels of total cholesterol, triacylglycerol, and nonesterified fatty acids were determined using enzymatic colorimetric assays (Wako Chemical, Neuss, Germany). Glucose concentration was determined with the glucose oxidase method (Merck Diagnostic, Darmstadt, Germany). Levels of urea, creatinine, alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase, alkaline phosphatase were determined using a MEGA automatic analyzer with Merck Diagnostic (Darmstadt, Germany) reagents.

Table 1. Hepatic and renal function tests after 25 days of treatment (p.o.) with 5% bark infusion (500 mg dried bark/kg), essential oil (100 mg/kg), or dehydrocrotonin (DHC, 100 mg/kg) from *C. cajucara*, and placebo (H_2O and Tween 80) in hyperlipidemic transgenic mice[a].

Groups	H_2O	5% infusion	Tween 80	Essential oil	DHC
AST (UI/L)	161±38	122±38	152±20	217±37	252±70
ALT (UI/L)	64±21	82±14	72±20	114±33	49±9
ALP (UI/L)	320±55	345±47	467±95	437±33	442±85
Creatinine (mg/dL)	0.21±0.03	0.27±0.03	0.15±0.02	0.19±0.01	0.18±0.03
Urea (mg/dL)	47±6	43±2	52±4	32±3[b]	44±4

Note: [a] mice overexpressing apoCIII and CETP, and deficient in LDL receptor ($LDLr^{+/-}$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. The results are presented as mean ± S.E. (n = 7). [b] Tween vs. essential oil, $p < 0.05$.

Table 2. Body weight and fasting plasma levels of cholesterol (CHOL) and triglyceride (TG) levels and CETP activity after 25 days of treatment (p.o.) with 5% infusion (500 mg dried bark/kg), essential oil (100 mg/kg), or dehidrocrotonin (DHC, 100 mg/kg) from *C. cajucara*, and placebo (H_2O and Tween 80) in hyperlipidemic transgenic mice[a].

	H_2O	5% infusion	Tween 80	DHC	Essential oil
Body weight (g)	27±2.1	26±1.4	28±1.6	26±0.9	29±3.1
CHOL (mg/dL)	162±30	130±10	196±84	178±29	240±37
TG (mg/dL)	515±81	300±131	406±196	576±158	322±81
CETP (% CE transfer)	28±5	30±2	34±6	36±7	39±5

Note: [a] mice overexpressing apoCIII and CETP, and deficient in LDL receptor ($LDLr^{+/-}$). Mean ± S.E. (n = 4–6).

Fast protein liquid chromatography

Pooled plasma samples (250 μL) from treated and control CIII/CETP/$LDLr^{+/-}$ and $LDLr^{-/-}$ mice were fractionated on a Superose 6 column (Pharmacia Biotech, Upsala, Sweden) using tris-buffered saline, pH 7.2, as described elsewhere (Berti et al. 2001). Cholesterol was determined enzymatically in each fast protein liquid chromatography (FPLC) fraction using enzymatic colorimetric assays.

CETP activity assay

The CETP-mediated transfer of cholesteryl ester from human HDL_3, labeled with [^{14}C]-cholesteryl ester (CE) (Oliveira and Quintão 1996), to acceptor lipoproteins (human VLDL and LDL) was determined using the method described by Berti et al. (2001). Results were expressed as the percent of cholesteryl ester transferred from [^{14}C]-CE-HDL to VLDL + LDL.

Statistical analysis

The data are presented as mean ± S.E. The nonparametric Mann–Whitney U test (GraphPad Instat® software, version 3) was used to compare treated mice with their respective placebo groups. Differences were considered significant when $P < 0.05$.

Results

The hypercholesterolemic and hypertriglyceridemic CIII/CETP/$LDLr^{+/-}$ mice were treated with 5% bark infusion (500 mg/kg), water (control), DHC (100 mg/kg), essential oil (200 mg/kg), or placebo (Tween 80) by gavage for 25 days. Plasma biochemical tests for liver and kidney functions were conducted to monitor for possible toxic effects. As shown in Table 1, there were no significant increases in plasma alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, or creatinine plasma concentrations after 25 days of treatment with any *C. cajucara* bark preparation. Table 2 shows the body weight, plasma lipid levels, and CETP activity in these hyperlipidemic mice after the treatments. Bark infusion reduced plasma levels of triglycerides by 42% and cholesterol by 20%, whereas essential oil reduced triglycerides by 20%. However, none of these changes reached statistical significance. DHC treatment had no effect on the plasma lipid levels. None of the treatments modified the plasma CETP activity.

Because the infusion seemed to be most effective in changing plasma lipid concentrations, further studies were carried out with bark infusion using two mouse models: combined hyperlipidemic (CIII/CETP/$LDLr^{+/-}$), and isolated hypercholesterolemic LDL-receptor knockout mice ($LDLr^{-/-}$). Body, liver, and perigonadal adipose tissue weights did not change significantly after 25 days of *C. cajucara* bark infusion treatment in either mouse model (data not shown). Table 3 shows plasma lipid, glucose, and CETP levels in nontransgenic wild-type mice (as reference values) and in both groups of hyperlipidemic mice treated with water (control) or 5% bark infusion. Plasma levels of cholesterol, free fatty acids, and glucose were not affected by the *C. cajucara* infusion in either mouse model. However, triglyceride levels in the combined hyperlipidemic (CIII/CETP/$LDLr^{+/-}$) mice were significantly reduced (–37%) by bark infusion treatment, as suggested by the first study (Table 2).

Total cholesterol levels were unaltered by the bark infusion treatment, but the distribution of cholesterol in the plasma lipoproteins was different, and may be more relevant to atherosclerosis risk than to the total cholesterol level. Previous studies in mice and rats have used the apoB precipitation procedure and the Friedwald (Friedwald et al. 1972) formula to calculate plasma lipoprotein fractions. Because

Table 3. Fasting plasma levels of triglycerides (TG), free fatty acids (FFA), cholesterol (CHOL), glucose (GLUC), and CETP activity in hyperlipidemic mice treated with 5% infusion of *C. cajucara* or water (control).

	Nontransgenic	CIII/CETP/LDLr$^{+/-}$		LDLr$^{-/-}$	
	Control	Control	Treated	Control	Treated
Number of mice	10	10	11	7	8
TG (mg/dL)	93±12	574±99	360±82[a]	144±14	158±17
FFA (mmol/L)	1.3±0.2	4.0±0.3	4.0±0.1	1.1±0.07	1.0±0.07
CHOL (mg/dL)	74±10	142±13	142±12	216±13	208±10
GLUC (mg/dL)	71±7	101±5	103±6	75±6	70±6
CETP (% CE transfer)		25±3	31±3		

Note: Mean ± S.E. [a] Mann Whitney U test: $p < 0.05$. Nontransgenic data are presented as reference values.

Fig. 1. Lipoprotein cholesterol distribution in hyperlipidemic mice treated with 5% infusion of *C. cajucara* or water (control). A: combined hyperlipidemic mice in which apoCIII and CETP are overexpressed and the LDL receptor is deficient. B: Primary hypercholesterolemic, LDL-receptor gene knockout (LDLr$^{-/-}$) mice. ABS, absorbance.

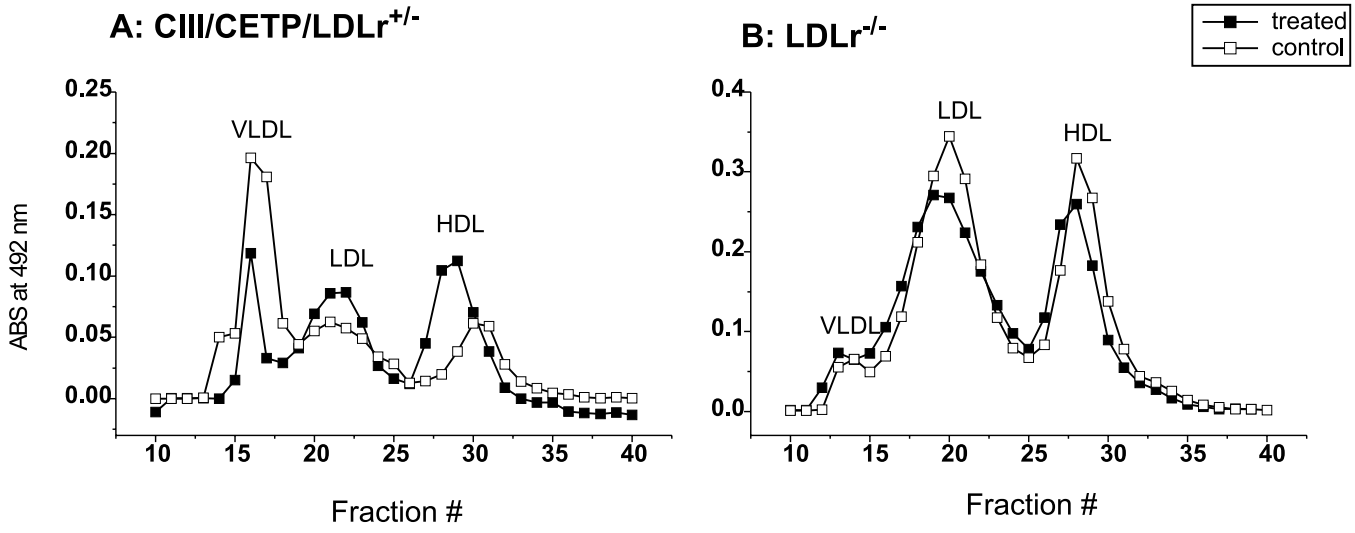

Table 4. Cholesterol concentration and percent distribution in the plasma lipoprotein fractions from hyperlipidemic mice treated with *C. cajucara* infusion or water (control).

				(VLDL + LDL)
Genotypes	VLDL mg/dL (%)	LDL mg/dL (%)	HDL mg/dL (%)	HDL
CIII/CETP/LDLr$^{+/-}$				
Control	60±1.4 (46)	42±14 (30)	31±7 (24)	3.3
Treated	29±12 (29)[a]	37±6.7 (36)	39±16 (35)[a]	1.7
LDLr$^{-/-}$				
Control	10±2.5 (4)	123±21 (56)	87±13 (40)	1.5
Treated	12±0.7 (6)	111±11 (54)	84±19 (40)	1.5

Note: Mean ± S.E. (n = 3–6 pools of samples). Values were calculated as the area under the peaks of FPLC profile, shown in Fig. 1. [a] Mann Whitney U test: $p < 0.05$ vs. respective control.

these species have low levels of LDL-cholesterol and apoE-rich HDL, the precipitating reagents may interact with and precipitate some HDL particles, skewing the results. In our study, the FPLC method was used. As shown in Fig. 1 and Table 4, *C. cajucara* bark infusion treatment promoted a redistribution of cholesterol among the lipoprotein fractions in combined hyperlipidemic mice. There was a marked reduction in VLDL fraction and an increase in HDL, with no net change in total cholesterol levels. The ratio of the apoB-containing lipoproteins (VLDL + LDL) to HDL was markedly reduced. No significant effects on the lipoprotein cholesterol distribution were observed in the plasma of hypercholesterolemic (LDLr$^{-/-}$) mice.

Discussion

The genetically modified mouse models used in this study mimic two types of human genetic hyperlipidemias. The

more common, moderate, combined hyperlipidemia results from the overexpression of two genes, apoCIII and CETP, and the underexpression of one gene, the LDL receptor. Overexpression of apoCIII reduces the plasma removal rate of triglyceride-rich lipoproteins, VLDL, and IDL (Aalto-Setala et al. 1992, 1996). The presence of CETP (not normally expressed in mice) is responsible for reducing the HDL levels; it promotes the transfer of cholesteryl ester from HDL to the other lipoproteins (Tall 1995). Partial or total deficiency in the expression of the LDL receptor ($LDLr^{+/-}$ and $LDLr^{-/-}$, respectively) leads to increased plasma LDL-cholesterol concentration, and is the defect in human primary hypercholesterolemia (Goldstein and Brown 2001). Therefore, these mice were used to screen potential hypolipidemic compounds.

In this study, we demonstrated that chronic treatment with organic extracts of *C. cajucara*, one containing DHC and the other containing essential oil, had no significant effect on the plasma lipid levels of genetic hyperlipidemic mice, whereas the aqueous bark infusion treatment reduced the plasma triglyceride concentration in the hypertriglyceridemic mice. High plasma levels of triglyceride-rich lipoproteins and their remnants are independent risk factors for coronary heart disease (Malloy and Kane 2001). Clinical trials have shown that reducing plasma triglyceride levels by 20%–50% (Miller 2000) decreases coronary heart disease events (Faergeman 2000).

Neither glucose nor free fatty acids plasma concentrations were altered by the bark infusion treatment, indicating that glucose homeostasis and adipose tissue lipolysis are not modified in these normoglycemic-hyperlipidemic mice (both genotype groups, CIII/CETP/$LDLr^{+/-}$ and $LDLr^{-/-}$).

Treatment with bark infusion altered the cholesterol distribution in lipoprotein fractions, reducing VLDL-cholesterol and increasing HDL-cholesterol in the combined hyperlipidemic mice. These effects were not due to the activity of CETP, because plasma CETP activity was not modified by any of the treatments. Reduced hepatic lipase activity or increased peripheral lipoprotein lipase activity could have increased HDL levels (Nunes et al. 2001; Braun and Severson 1992) or increased the conversion of VLDL in LDL. The observed decrease in total plasma triglyceride levels suggests increased intravascular lipoprotein lipase activity, which may have been downregulated by apoCIII, a known inhibitor of this enzyme (Brown and Baginsky 1972). A third mechanism that may have contributed to the decrease in the VLDL fraction is a lower hepatic VLDL secretion rate. Accordingly, Silva et al. (2001*c*) have shown that DHC acutely reduced hepatic triglyceride secretion when lipoprotein lipase was inhibited by Triton WR1339.

The effect on lipoprotein cholesterol fractions by the *C. cajucara* bark infusion treatment may favorably affect the risk of atherosclerosis; the ratio between the atherogenic lipoproteins (VLDL + LDL) and the antiatherogenic HDL was reduced by half (Table 4) in treated mice.

Although several studies (Farias et al. 1996; Silva et al. 2001*a*, 2001*b*, 2001*c*) have reported hypolipidemic effects of the *C. cajucara*-derived DHC fraction, these effects may be related to the nongenetic causes of the hyperlipidemias in those models (i.e., diet, ethanol, and drugs). Our work shows that DHC and essential oil were ineffective in treating hyperlipidemia of genetic origin.

Conclusions

In conclusion, we have shown that treatment of genetically combined hyperlipidemic mice with aqueous infusion of *C. cajucara* bark reduced plasma triglycerides and promoted a redistribution of cholesterol among lipoprotein fractions, in a way that may reduce the risk factors for atherosclerotic diseases. It remains to be elucidated which specific compound, or combination of compounds, from *C. cajucara* is responsible for these effects.

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