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PAPER

Chloroquine increases low-density lipoprotein removal from plasma in systemic lupus patients

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Low-density lipoprotein (LDL) pathway in systemic lupus erythematosus (SLE) patients taking chloroquine diphosphate (CDP) was evaluated through the kinetic behavior of a radioactive cholesterol-rich nanoemulsion (LDE) that resembles the LDL lipidic structure. LDE was labeled with ^{14}C-cholesteryl ester (^{14}C-CE), then IV injected in inactive female SLE patients: 10 taking CDP (CDP), 10 without therapy (NO THERAPY); and 10 normal subjects (CONTROL). Groups were age-matched and followed rigorous selection criteria of conditions that interfere in the lipid profile. Blood samples were collected in pre-established intervals after infusion for radioactivity measurement. Fasting lipoproteins were determined in the beginning of kinetic studies. Fractional clearance rate (FCR) of ^{14}C-CE was significantly different in the three groups ($P = 0.03$). In fact, a greater FCR of ^{14}C-CE was observed in CDP compared to NO THERAPY (0.076 ± 0.037 versus $0.046 \pm 0.021\,h^{-1}$; $P < 0.05$) and to CONTROL ($0.0516 \pm 0.0125\,h^{-1}$; $P < 0.05$). Accordingly, a significant lower total and LDL cholesterol were observed in CDP (156 ± 16 and 88 ± 16 mg/dl) compared to NO THERAPY (174 ± 15 and 108 ± 17 mg/dl; $P < 0.05$) and to CONTROL (200 ± 24 and 118 ± 23 mg/dl; $P < 0.05$). In contrast, no difference in (FCR) of ^{14}C-CE of NO THERAPY and CONTROL groups was observed. This is the first *in vivo* demonstration that LDE removal by LDL receptor from plasma is increased in SLE patients taking CDP with a consequent beneficial decrease in LDL-c levels. *Lupus* (2007) **16**, 273–278.

Key words: atherosclerosis; chloroquine; dyslipoproteinemia; emulsions; lipoproteins; low-Density lipoprotein; systemic lupus erythematosus

Introduction

Antimalarial drugs containing the four aminoquinilone radicals have a modulating effect on the inflammatory process in rheumatic diseases.[1–3] In fact, hydroxychloroquine (HCQ) and chloroquine diphosphate (CDP) are widely used in systemic lupus erythematosus (SLE).[4–8]

Furthermore, antimalarials have a plasma lipid lowering effect in SLE[9–11] that is therapeutically relevant due to the increased risk of premature atherosclerosis in this disease.[12] In fact, dyslipidemias are very frequent in SLE[12–14] and certainly play a pivotal role in the 50 times greater risk of developing coronary artery disease (CAD),[15] an important cause of mortality in SLE.[12,16,17]

SLE dyslipoproteinemias are known to be aggravated by disease activity[18–21] and therapy.[22–24] The latter is characterized by an increased total and LDL cholesterol, a dyslipidemia pattern observed in more than half of lupus patients.[12–14] Importantly, LDL is recognized as one of the main risk factor for atherosclerosis[25] and therefore the reported beneficial effect of antimalarials in reducing this lipoprotein[9,10,26–31] is of great interest.

Since LDL removal by its specific receptor is highly effective in decreasing this lipoprotein fraction,[32] we evaluated the LDL pathway in lupus patients with or without CDP and in controls through the determination of plasma kinetics of a radioactively labeled artificial cholesterol-rich nanoemulsion (LDE) that resembles LDL lipidic structure.[33–35] LDE has been used as a surrogate for native LDL to investigate in clinical studies the process of LDL removal from plasma.[36–38]

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Patients and methods

Study population

Twenty consecutive female SLE patients were selected in the Outpatient Lupus Clinics at the Rheumatology Division of the University of São Paulo. All patients were under 50 years old and fulfilled four or more of the revised American College of Rheumatology criteria for the classification of SLE.[39] SLE patients were divided in two age-matched groups according to current therapy at the time of evaluation: CDP – exclusive use of 250 mg of CDP daily for at least one year; and NO THERAPY – inactive disease without any CDP drug therapy or have stopped this drug for at least one year. SLE patients were not under corticosteroids nor immunosuppressive drugs in the last year, and none of them were in use of any lipid-raising drugs, such as anti-convulsants, anti-hypertensives (beta blockers and diuretics), estrogen and/or progesterone containing agents, or lipid-lowering agents. Exclusion criteria were presence of clinical and/or biochemical evidence of Diabetes Mellitus, CAD, liver and thyroid disease, and history of alcohol use. No patient was pregnant or menopausal. All patients had serum creatinine levels below 1.5 mg/dl and proteinuria less than 0.3 g/L/day. Clinical activity was measured according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[40] and all SLE patients have inactive disease with SLEDAI scores of 0. Ten healthy and age-matched female subjects (CONTROL) were selected for comparison and were studied in parallel with lupus patients. None of the SLE patients and controls had a body mass index (BMI) greater than 30 kg/m^2.

LDE preparation

LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphotidylcholine, 1 mg triolein and 0.5 mg cholesterol purchased from Nu-Check Prep (Elysian). [^{14}C] cholesteryl ester purchased from Amersham International (Amersham, UK) was added to the mixture. The emulsification of lipids by prolonged ultrasonic irradiation in aqueous media and the two-step ultracentrifugation procedure of the crude emulsion with density adjustment by addition of KBr to obtain LDE microemulsion was carried out by the method of Ginsburg[41] modified by Maranhão *et al.*[33] LDE was dialysed against saline solution and passed through 0.22 μm filter for injection into the patients.

LDE plasma kinetics

The participants were fasting for 12 h at the beginning of the test at approximately 8:30 a.m., but they were allowed two standard meals (total of 1800 kcal) during the study at ~12:30 p.m. and 7 p.m. LDE containing 37 kBq [^{14}C]cholesteryl ester in a total lipid mass of 5–6 mg (in a 200 μl volume) was intravenously injected in a bolus. Plasma samples were collected during 24 h, in intervals of 5 min, 1, 2, 4, 6, 8 and 24 h after the injection. Aliquots (1 ml) of blood plasma were transferred to counting vials containing 7.0 ml scintillation solution (ULTIMA GOLD XR, Packard Bioscience, Meriden, USA). Radioactivity was counted using a Packard 1660 TR (Meriden, USA) Liquid Scintillation Analyser.

Calculation of FCR of the emulsion radioactive lipids

FCR of the LDE [^{14}C]cholesteryl ester, was calculated according to the method described by Matthews as FCR, where a_1, a_2, b_1 and b_2 were estimated from biexponential curves obtained from the remaining radioactivity found in plasma after injection, fitted by least squares procedure:

$$y = (a_1 . e^{-b1t}) + (a_2 . e^{-b2t}),$$

where y represents the radioactivity plasma decay in function of time (t); a indicates the linear coefficient and b, the angular coefficient, which represents the FCR in h^{-1}. The FCR were estimated from parameters a_1, b_1 and b_2 by the following equation: $FCR = (a_1/b_1 + a_2/b_2)^{-1}$. Calculations were performed by compartmental analysis using the ANACOMP software.[42]

Informed consent and radiological safety

The experimental protocol was approved by the local Ethical Committee and a written informed consent was given by all participants. A pregnancy test was performed one day before the kinetic study and all tests were negative. The safety of the radioactive dose intravenously injected into the patients was assured according to the regulations of the International Commission on Radiological Safety as described in our previous study.[43] The equivalent absorbed dose on each experiment was 0.03 mSV which is below the 50 mSV annual limit for intake of radionuclides.

Lipid profile

All SLE patients and controls were continued with their normal diet and were fasting for at least 12-h at the beginning of the study. Fasting lipoproteins were determined on the same day of the kinetic studies. Plasma total cholesterol (TC) and triglycerides (TGs) were measured enzymatically (Boehringer Mannheim,

Argentina and Merck, Germany, respectively) on a RA 1000 analyser (Technicon Instruments Corp).[44,45] High density lipoprotein cholesterol (HDL-c) was obtained after precipitation of very LDL cholesterol (VLDL-c) and LDL cholesterol (LDL-c) by phosphotungstic acid and magnesium chloride.[46] VLDL-c and LDL-c were estimated since all subjects had TGs less than 400 mg/dl.[47] VLDL-c levels were calculated from the division of serum TG by 5 (TG/5).[47] LDL-c levels were estimated using the equation:[47]

$$TC = HDL\text{-}c + TG/5 + LDL\text{-}c$$

Statistical analysis

Results are presented as the mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) and if a significant result was obtained ($P < 0.05$), Tukey's test was performed to evaluate differences between groups. Correlations among plasma lipid fractions and FCRs were calculated using Spearmann's rank correlation test. Statistical significance was set as $P < 0.05$.

Results

The distribution of age, weight, height and BMI of the three groups is shown in Table 1. As expected, age was similar in CDP, NO THERAPY and CONTROL groups ($P = 0.93$). In addition, no significant difference was found between groups concerning weight ($P = 0.39$) and height ($P = 0.96$). Likewise, BMI did not significantly differ between groups ($P = 0.31$). SLE patients of CDP group were in use of this drug for 7.1 ± 2.9 years and had a similar disease duration compared to NO THERAPY (9.5 ± 4.6 and 8.2 ± 6.6 years, respectively, $P = 0.10$). Importantly, SLE patients of both groups had a SLEDAI score of zero.

Table 1 Demographic features, disease duration and SLEDAI in SLE groups (CDP and NO THERAPY) and CONTROL

Group	*CDP* (n = *10*)	*NO THERAPY* (n = *10*)	*CONTROL* (n = *10*)	P
Age (years)	35.4 ± 7.5	36.5 ± 6.9	35.6 ± 8.9	0.93
Weight (kg)	60.2 ± 6.8	63.0 ± 8.8	57.5 ± 10.4	0.39
Height (m)	1.61 ± 0.04	1.60 ± 0.06	1.60 ± 0.10	0.96
BMI (kg/m^2)	23.0 ± 2.5	24.3 ± 2.4	22.2 ± 3.7	0.31
Disease duration (years)	9.5 ± 4.6	8.2 ± 6.6	–	0.10
SLEDAI	0	0	–	–

Values were expressed in mean ± SD; BMI = body mass index; SLEDAI = systematic lupus erythematosus disease activity index.

The lipoprotein profile of CDP, NO THERAPY and CONTROL are shown in Table 2. CDP had lower LDL-c levels compared to NO THERAPY (88 ± 16 versus 108 ± 17 mg/dl; $P < 0.05$) and CONTROL (88 ± 16 versus 118 ± 23 mg/dl; $P < 0.05$). Similarly, TC levels in CDP (156 ± 16 mg/dl) were lower compared to NO THERAPY (174 ± 15 mg/dl; $P < 0.05$) and healthy controls (200 ± 24 mg/dl; $P < 0.05$). In contrast, higher HDL-c levels were found in CDP compared to NO THERAPY (54 ± 8 versus 46 ± 9 mg/dl; $P < 0.05$) although no difference was observed compared to the controls ($P > 0.05$). In addition, NO THERAPY group also had lower HDL-c compared to healthy controls, as well as a lower TC levels ($P < 0.05$). TG and VLDL-c tended to be lower in CDP compared to the other two groups, although this difference did not reach statistical significance ($P = 0.06$ and $P = 0.06$, respectively).

LDE plasma kinetics

Figure 1 shows the plasma decaying curves of the radioactively labeled LDE cholesteryl ester during the 24 h of the study. It should be noticed that the curve obtained in CDP group was pronouncedly faster than NO THERAPY and CONTROL curves (Figure 1). Indeed, as shown in Table 3, the [^{14}C]cholesteryl ester FCR was significantly different in the three groups studied ($P = 0.03$). In fact, the [^{14}C]cholesteryl ester FCR in CDP group was greater compared to NO THERAPY ($P < 0.05$) and to CONTROL ($P < 0.05$). In contrast, the [^{14}C]cholesteryl ester FCR was similar in the comparison of NO THERAPY and CONTROL ($P > 0.05$).

Discussion

This is the first *in vivo* demonstration that in SLE patients treated with CDP the LDE plasma kinetics is accelerated with consequent decrease in LDL cholesterol.

Table 2 Plasma lipid profile of SLE groups (CDP and NO THERAPY) and CONTROL

Parameter	*CDP* (n = *10*)	*NO THERAPY* (n = *10*)	*CONTROL* (n = *10*)	P
Cholesterol				
Total	156 ± 16[a,b]	174 ± 15[a]	200 ± 24	<0.001
HDL-c	54 ± 8[b]	46 ± 9[a]	64 ± 7	<0.001
LDL-c	88 ± 16[a,b]	108 ± 17	118 ± 23	<0.001
VLDL-c	13 ± 4	20 ± 7	17 ± 7	0.06
TGs	68 ± 19	103 ± 38	86 ± 33	0.06

Values expressed in mean ± SD, in mg/dl.
[a]$P < 0.05$ versus CONTROL.
[b]$P < 0.05$ versus NO THERAPY.

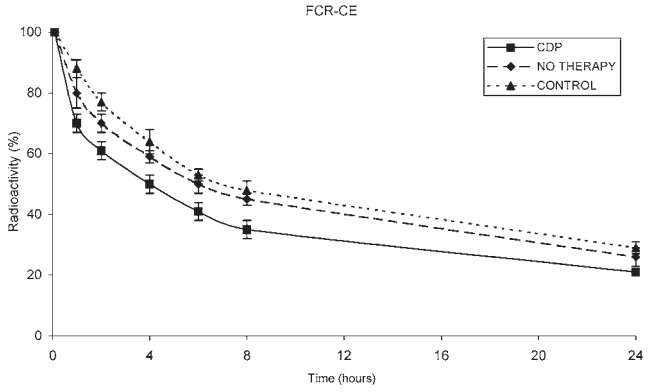

Figure 1 Disappearance curves of the emulsion [^{14}C]cholesteryl ester of CDP, NO THERAPY and CONTROL groups. LDE clearance in CDP patients (continuous line), NO THERAPY patients (dashed line) and CONTROL subjects (dotted line) during 24 hs after intravenous infusion. Disappearance curves of the emulsion [^{14}C]cholesteryl ester of CDP were faster than those of NO THERAPY and CONTROL ($p = 0.03$), indicating a significant increase of clearance in SLE patients taking CDP.

To achieve these findings, rigorous selection criteria was established to eliminate the main systemic diseases that promote specific alterations on lipid metabolism.[48–52] Additionally, the progressive increase in total and LDL cholesterol with aging[53] and menopause[54] was avoided by the exclusion criteria. All patients included in the study had an inactive form of the disease since it is known that activity can aggravate lupus dyslipoproteinemia[19] and were not using drugs that could interfere in lipoprotein metabolism.[55] Also importantly, the CDP administration period was long enough to assure that the changes in lipid metabolism by CDP were sustained, which occurs after one year of treatment.[31,56]

The great advantage of the LDE system used herein is to specifically evaluate the *in vivo* function of LDL receptors that remove the lipoprotein from plasma into cells. LDE is made without protein but when injected into the circulation and in contact with plasma it acquires several small molecular weight apolipoproteins (apo), including apo E.[33] Apo E endows LDE particles to bind to the LDL receptors, since those receptors recognize not only the apo B present in LDL-c, but also apo E that is not found in the LDL fraction.[35,57] Apo E binds to the LDL receptor with a greater affinity than apo B100 and is thus removed faster than the native LDL.[35,57] Moreover, the known differences in LDL sub-classes among subjects[58–60] which may influence the removal of the lipoprotein from the plasma are excluded by the present approach since LDE is a standard preparation allowing a more accurate definition of receptor function.[37,38]

Table 3 FCR of [^{14}C]cholesteryl ester in SLE groups (CDP and NO THERAPY) and CONTROL

FCR	*CDP*	*NO THERAPY*	*CONTROL*	P
[^{14}C] cholesteryl ester	$0.076 \pm 0.037^{a,b}$	0.046 ± 0.021	0.051 ± 0.012	0.03

Values in h^{-1}, expressed in mean $\pm$ SD.
[a]$p < 0.05$ versus CONTROL.
[b]$p < 0.05$ versus NO THERAPY.

Of interest, accelerated LDE removal has been described for acute mielocytic leukemia[61,62] and a defective function was detected in familial hypercholesterolemia.[37] In contrast, we identified herein for the first time that LDL receptor function is normal in SLE supported by the similar LDE removal in lupus patients without therapy and healthy controls. As expected, LDL levels in these two groups were alike in accordance with previous report in inactive untreated lupus patients.[19]

Our *in vivo* study also identified that CDP up-regulation of LDL-receptor is a very efficient mechanism for LDL reduction in SLE. A possible explanation for our findings is the known inhibitory effect of chloroquine in lysosomal hydrolysis of cholesterol ester reducing the release of repressive cholesterol with consequent increase in LDL receptor synthesis, as has been demonstrated by *in vitro* experiments in fibroblast and smooth-muscle cells.[63,64] Reinforcing this hypothesis, the approximately 50% greater LDE removal from the plasma in our CDP was paralleled by a 20% reduction in the LDL-c level compared to untreated patients. Indeed, a decrease in LDL-c levels was also identified in randomized trials after the use of hydroxychloroquine in diabetic patients.[65,66]

In SLE, it has previously been demonstrated that hydroxychloroquine use elicits lower LDL cholesterol levels, particularly in patients taking corticosteroids.[9,10] In this regard, the known increase in lipoprotein hepatic synthesis induced by steroids[10,13,67–69] may be counterbalanced by the antimalarial enhanced LDL removal. Remarkably, statins (or HMG-CoA) also increase the removal from the plasma of both native LDL and LDE[37,38] in addition to inhibiting the rate-limiting step of cholesterol synthesis.[70]

Our data provide consistent evidence that CPD powerfully influence the status of LDL receptor function, a known determinant of LDL levels,[32] emphasizing its use in lupus patients with high LDL levels, particularly those taking steroids. The possible synergistic effect between CDP and statins should be explored in future studies.

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