Plasma purification is associated with reduced inflammation factors and down-regulation of lipid metabolic and ER stress proteins in patients with hyperlipidemia

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Research

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Abstract

Background Hyperlipidaemia is a strong risk factor for arteriosclerosis and cardiovascular disease (CVD). Therapy with lipid-lowering drugs often results in many side effects. The aim of our study was to investigate the potential effects of non-drug therapy with double-filtration plasmapheresis (DFPP) on lipid metabolism-, endoplasmic reticulum (ER) stress- and apoptosis-related proteins in peripheral blood mononuclear cells (PBMCs) before and after lipid clearance in patients with hyperlipidaemia.

Methods Thirty-five patients with hyperlipidaemia were selected for this study. Proteins related to lipid metabolism [CD36, proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDL receptor], ER stress (GRP78, CHOP, ATF4, EIF2a) and apoptosis (Bcl-2, BAX, and Caspase-3) were assayed by western blot, reactive oxygen species (ROS) were measured by flow cytometry (FCM), and serum inflammatory factors were detected by ELISA.

Results Compared with their pre-DFPP values, the values of most lipid metabolic parameters, such as cholesterol, triglycerides, LDL, lipoprotein a [Lp(a)] and small dense LDL cholesterol (sdLDL), were reduced after DFPP. DFPP was associated with the downregulation of proteins related to lipid metabolism, ER stress and apoptosis, which also resulted in decreased ROS and serum inflammatory factor release.

Conclusion DFPP has lipid-lowering activity and can also regulate lipid metabolism-, ER stress- and apoptosis-related proteins in PBMCs and reduce the levels of inflammatory factors in patients with hyperlipidaemia (ClinicalTrials.gov number: NCT03491956).

Introduction

Hyperlipidaemia is one of the most important factors associated with cardiovascular disease (CVD) and often results in fatty liver, cerebral thrombosis and/or infarction, and severe pancreatitis [1-4]. However, there is low awareness of the risk of CVD, and the benefits of treatment are underappreciated in the general population. Lipid disorders are present in 40.4% of the adult population in China, with hypercholesterolemia present in 4.9% of the population, hypertriglyceridemia present in 13.1% of the population, and high-low-density lipoproteinemia (LDL) present in 33.9% of the population [5]. Therefore, there is an urgent need for prevention and treatment of increased serum lipid concentrations.

One important marker of hyperlipidaemia is abnormal expression of lipid-related metabolic proteins. CD36 is a lipid transporter and multifunctional scavenger receptor [6]. It plays roles in modification of LDL and uptake of modified lipoproteins, which causes macrophage lipid accumulation, leading to the formation of foam cells [7]. In hyperlipidaemia patients, CD36 expression was abnormal, and increased CD36 expression could result in endoplasmic reticulum (ER) stress, macrophage apoptosis, insulin resistance and CVD [8]. Another important lipid metabolic protein, proprotein convertase subtilisin/kexin type 9 (PCSK9), is mainly expressed in the liver, intestinal tract and kidney. In the context of decreased PCSK9 activity, LDL receptor (LDLR) is upregulated, resulting in a decrease in LDL cholesterol levels,

potentially reducing the risk of CVD [9]. Previous studies have indicated that PCSK9 levels were associated with metabolism-related indices, such as fasting blood glucose, insulin, triglycerides and liver triglyceride content [10]. Alirocumab, a PCSK9 inhibitor, has been approved by the US Food and Drug Administration (FDA) in the treatment of patients with hypercholesterolemia. In another study, Alirocumab was found to reduce or improve cardiovascular outcomes after acute coronary syndrome [11]. Previous studies have indicated that PCSK9 and CD36 are associated with lipid metabolism and CVD outcomes [12]. However, it is unclear whether lipid plasmapheresis could affect CD36, PCSK9 and LDLR.

Statins are the most frequently used drug for the treatment of hypercholesterolemia. However, adverse reactions, such as rhabdomyolysis, abnormal liver enzymes and gastrointestinal symptoms, frequently prevent long-term use of this medication. Moreover, a meta-analysis indicated that statins were associated with an increased risk of insulin resistance and new-onset diabetes [13]. Similarly, Ezetimibe shows the same side effects as statins [14]. Some patients are reluctant to take lipid-lowering drugs considering the adverse effects of statins, and some are worried about control of blood glucose, especially among patients with diabetes.

Non-drug therapy to reduce lipid levels has been used for many years. Heparin-induced extracorporeal LDL precipitation (HELP) was proven to be capable of reducing lipid levels and the risk of CVD in patients with familial hypercholesterolemia in a previous study; however, the high costs of consumption and treatment prevented its further large-scale clinical usage. Instead, double-filtration plasmapheresis (DFPP) has recently replaced HELP in the treatment of familial hypercholesterolemia, hyperlipidaemia-induced severe acute pancreatitis, and coronary artery disease after coronary artery stenosis [15-18]. DFPP has the advantages of simple use for operators, safety for patients, a low price (7000 RMB, equal to 1000 US dollars/session, can be covered by Medicare), a short treatment time (3-4 hours) and no plasma or albumin replacement.

DFPP technology has been used to treat hyperlipidaemia patients at Pudong Medical Center since 2018. The aim of this study was to investigate the lipid removal effect of DFPP and the association of DFPP with changes in lipid metabolism- and ER stress-related proteins in PBMCs before and after treatment in patients with hyperlipidaemia.

Materials And Methods

Study participants

Patients who had hyperlipidaemia [diagnosed with hypercholesterolemia (cholesterol>5.18 mmol/L) or hypertriglyceridemia (triglycerides>2.3 mmol/L), mixed hyperlipidaemia (cholesterol>5.18 mmol/L and triglycerides>2.3 mmol/L), and high low-density lipoproteinemia (low-density lipoprotein>3.4 mmol/L) according to the 2019 guidelines of the European Society of Cardiology] with no other diseases were selected for this study. No statins or other lipid-lowering drugs were prescribed for the patients. All patients provided and signed informed consent. The study was approved by the Ethical Committee of Shanghai Pudong Hospital (Fudan University, Pudong Medical Center, Shanghai, China), and experiments

were performed in accordance with the Ethical Committee's guidelines and regulations. The ClinicalTrials.gov number was NCT 03491956.

Exclusion criteria: Patients were excluded if (1) they had coexisting diabetes or hypertension; (2) they had tumours, liver and kidney dysfunction, severe oedema, cardiac insufficiency, respiratory insufficiency caused by severe lung disease, or pregnancy; and (3) they were over 80 years old.

Treatment procedure

Plasmapheresis using a Plasauto iQ automatic blood purification system, model KM-9000 (Kawasumi, Tokyo, Japan), was performed in this study, with a PE-08 primary membrane plasma separator and an EC-4A20 secondary membrane plasma component separator. Cubital elbow median veins on both arms were selected for artery and vein access. The blood flow was 60-100 mL/min, the plasma separation rate was 30% of the blood flow, and the plasma rejection rate was 15% of the plasma separation rate. Ordinary heparin was used as an anticoagulant, with an initial dose of 3000 U, followed by an additional maintenance dose of 20-40 U/(kg·h). The therapeutic target was calculated as weight (kg) × 40 mL (1-1.4 times blood volume). Each session lasted for 3-4 hours.

Laboratory examination

Isolation of PBMCs

Blood samples were taken from patients with hyperlipidaemia, and acidic citrate dextrose was added as an anticoagulant. To isolate PBMCs, the blood samples were diluted with PBS, and lymphocyte separation medium was added (Hao Yang Biological Manufacture Co., Tianjin, China). The samples were centrifuged at 1000 rpm for 40 minutes, the middle cell layer was collected, and PBMCs were obtained after washing three times with PBS.

Detection of lipid metabolism-related, ER stress-related and apoptosis-related proteins

The proteins subjected to western blotting were extracted using lysis buffer (Invent, China), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The membrane carrying the protein bands was blocked in Tris-buffered saline with Tween (TBST) containing 5% skim milk for 1 hour and incubated with primary antibodies at 4 °C overnight. After washing, the membrane was incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. Secondary antibodies included goat anti-rabbit IgG and goat anti-mouse IgG. After washing, the membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA). Protein signals were captured using a Bio-Rad ChemiDoc™ XRS system (Bio-Rad, Hercules, CA, USA). Data were quantified by Quantity One software (Bio-Rad, CA, USA) [19]. Primary antibodies against LDLR, CD36, PCSK9, GRP78, CHOP, ATF4, p-EIF2, Bcl-2, BAX and Caspase-3 and secondary antibodies were obtained from Proteintech (Proteintech Group, Inc., IL, USA), and an antibody against EIF2 was purchased from CST (Danvers, MA, USA).

Detection of ROS and serum inflammatory factors

Nonstimulated PBMCs were detected with a ROS assay kit (Beyotime, China) after isolation. PBMCs in each sample were washed twice with PBS. The fluorescent probe used to determine the level of ROS in PBMCs was diluted to a concentration of 10 $\mu\text{mol/L}$. PBMCs were suspended in the diluted fluorescent probes and incubated in a plate at 37°C for 20 minutes. The PBMCs were washed three times with cell culture medium to remove probe that did not enter the cells. Finally, the ROS level was detected by flow cytometry (BD, USA). Serum inflammatory factors (IL-1b, IL-6, TNF-a) were detected by ELISA using commercial kits (Neobioscience, Shanghai, China).

Statistical analysis

Data are expressed as the means $\pm$ SDs, with the exception of skewed data, which are expressed as the median (range). After correcting non-normally distributed data, the differences were analysed with a paired t test, and a *P* value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 22.0 statistical software (IBM, IL, USA).

Results

Clinical characteristics of the patients

The age, sex, weight and baseline lipid profiles of the patients are shown in Table 1. The mean serum total cholesterol post DFPP was 2.4 [1.8, 3.1] mM, which represented a significant decrease compared to the pre-DFPP values. Serum LDL and triglycerides post DFPP showed the same trend. Compared with the values measured before DFPP, Lp(a) and sdLDL values also showed an obvious decrease post DFPP. These data indicated that DFPP could remove blood lipid molecules effectively in 3-4 hours.

Table 1 The variations of lipids before and after DFPP treatment.

	Pre-DFPP (N=35)	Post-DFPP (N=35)
Age(years)	51[44,61]	-
Male	12	-
Weight (kg)	73.1 [68.4,83.9]	-
Total cholesterol (mM)	8.2 [6.6,10.0]	2.4 [1.8,3.1]**
Triglyceride (mM)	8.9 [7.1,10.2]	2.1 [1.5,3.1]**
LDL-C (mM)	4.7 [3.9,5.5]	1.4 [1.1,1.8]**
Lp(a) (mg/L)	187.3[137.9,200.7]	78.3 [53.8,104.8]**
sdLDL-C (mM)	2.1[1.8,2.7]	0.3 [0.2,0.5]**
Patients with hypercholesterolemia	18	-
Patients with hypertriglyceridemia	10	-
Patients with mixed hyperlipidemia	3	-
Patients with high low-density lipoproteinemia	4	-

Pre-DFPP: patients before DFPP treatment; Post-DFPP: patients after DFPP treatment; LDL-C, low density lipoprotein; Lp(a), lipoprotein(a). sdLDL, small dense low-density lipoprotein. Data were expressed as median (range). ** $P<0.01$ vs pre-DFPP.

DFPP was associated with changes in lipid metabolism-related proteins

The protein expression levels of lipid metabolism-related proteins assayed by western blot are shown in Fig 1. LDLR, PCSK9 and CD36 were all downregulated post DFPP. Our results indicated that DFPP may influence key lipid metabolism-related transport proteins.

DFPP reduced the expression of ER stress-related proteins

The expression of ER stress-related proteins (GRP78, CHOP, ATF4 and p-EIF2 α) is shown in Fig 2. Compared with the expression of these proteins prior to DFPP, GRP78, CHOP, ATF4 and p-EIF2 α showed a significant decrease post DFPP. These data demonstrated that DFPP was associated with reduced ER stress in hyperlipidaemia patients.

DFPP inhibited hyperlipidaemia-induced cell apoptosis-related protein expression

As Fig 3 shows, excessive lipids induced increased expression of apoptosis-related proteins in PBMCs, and DFPP treatment fully restored the expression of Bcl-2 and inhibited the expression of BAX and Caspase 3.

DFPP reduced ROS levels in PBMCs and alleviated inflammatory factors in serum

The ROS levels in hyperlipidaemia patients were significantly decreased compared with those of patients before DFPP (Fig 4). In addition, in Table 2, markers of inflammation showed an obvious decline after DFPP. These results showed that DFPP could effectively decrease ROS levels and reduce inflammatory factor release in hyperlipidaemia patients.

Table 2 DFPP reduces inflammation parameters

	Pre-DFPP (N=35)	Post-DFPP (N=35)
IL-1β (pg/mL)	17.9 [15.3, 20.1]	9.3 [8.1, 10.7]*
IL-6 (pg/mL)	16.0 [13.4, 20.8]	5.6 [4.1, 8.4]**
TNF-α (pg/mL)	21.7 [19.5,23.5]	9.3 [8.5,11.0]**

Pre-DFPP: patients before DFPP treatment; Post-DFPP: patients after DFPP treatment; Serum IL-6, TNF-α, IL-1β in patients with hyperlipidemia were detected by ELISA kit. Data were expressed as median (range). **P*<0.05 and ***P*<0.01 vs. Pre-DFPP.

Adverse events

One patient experienced a hypotensive episode during the procedure and quickly recovered after infusion of 0.9% normal saline. No other adverse events (blood coagulation, bleeding, or allergy) occurred during the treatment period.

Discussion

Our study indicates that DFPP could perfectly reduce the levels of lipids, such as LDL, cholesterol, triglycerides, Lp(a), and sdLDL-cholesterol, and this finding is similar to the results of other studies [15]. The safety, easy operation and low cost of DFPP indicate that it is suitable for widespread clinical use to reduce markers of hyperlipidaemia.

Changes in lipid metabolic proteins (CD36, PCSK9, and LDLR)

Patients with hyperlipidaemia often exhibit abnormal expression of lipid metabolic proteins. CD36 expression was increased in patients with hypercholesterolemia [20]. A previous study showed that CD36 promoted cell uptake of free fatty acids and regulated intestinal cholesterol synthesis [21]. Additionally, ox-LDL could induce platelet activation and ROS production, ultimately resulting in thrombosis via the

CD36 and MAPK pathways [22-24]. An in vivo study showed that insulin resistance and inflammatory factor release were reduced in CD36 knockout mice, indicating that CD36 plays an important role in humans [25]. CD36 is closely related to PCSK9, but in different organs, these factors behave differently. When PCSK9 was inhibited, CD36 expression increased in the intestine but decreased in the liver and glomerular podocytes [26]. PCSK9 could stimulate macrophages to take up ox-LDL and lead to atherosclerosis [27]. PCSK9 is a member of the proprotein convertase family of proteins, which regulate a variety of physiological and pathological processes, including lipid metabolism, inflammatory responses, glucose metabolism, apoptosis, and other processes [27]. In another report, it was found that PCSK9 might impair the function of LDLR, the LDL receptor. LDL is transported into cells via LDLR, which is closely related to the level of LDL in plasma. The deletion of the LDLR gene in familial hypercholesterolemia may be an important event in the pathogenesis of the disease [28]. A previous study showed that lipid plasmapheresis could reduce plasma PCSK9 levels [29]. Similarly, our study showed that lipid plasmapheresis could induce downregulation of the PCSK9 protein in isolated PBMCs. For the first time, the protein expression of CD36 and LDLR was shown to be obviously downregulated in PBMCs after lipid plasmapheresis. This result differs from the result obtained after therapy using statins. In patients subjected to statin therapy, PCSK9 and LDLR were upregulated, and CD36 was downregulated [30]. It is notable that LDLR was upregulated in patients using long-term statin and PCSK9 inhibitor therapy. These different results may be explained by differences in lipid clearance. The results also suggested that the removal of cholesterol and LDL from plasma by DFPP allows the body to maintain LDL levels in vivo and prevents LDL levels from dropping sharply, thus lowering LDLR and leading to a decrease in PCSK9 and CD36 levels in PBMCs. PBMCs are closely related to foam cell production, and PCSK9 and CD36 are also related to cardiovascular disease, so it is expected that lipid clearance via non-drug therapy will have broad applications in the treatment of hyperlipidaemia and reduce the risk of CVD.

Decrease in ER stress

ER stress is one of the mechanisms in hyperlipidaemia. The main functions of the endoplasmic reticulum include properly folding modified proteins, controlling cholesterol production, and storing intracellular Ca^{2+} [31]. A previous study indicated that PBMC ER stress and lipids were strongly associated with coronary artery disease [7, 32]. Insults interfering with PBMC ER function lead to the accumulation of unfolded and misfolded proteins in the ER, which initiates the unfolded protein response (UPR). When the UPR fails to control the level of unfolded and misfolded proteins, ER-initiated apoptotic signalling is induced. When accompanied by high blood lipid levels, endoplasmic reticulum stress is more harmful to the body. The ER stress-related protein GRP78 was upregulated in obese animals [31]. GRP78, a signature protein of ER stress, can bind to endoplasmic reticulum stress factors to maintain the endoplasmic reticulum in a non-stress state under normal conditions [33]. Protein kinase R-like ER kinase (PERK) is the initiating protein of the endoplasmic reticulum stress pathway, and CHOP is also an important protein that links upstream and downstream proteins. For the first time, the results obtained here indicated that DFPP may be associated with a decrease in ER stress in PBMCs, indicating that lipid plasmapheresis is effective in decreasing potential risk factors for cardiovascular disease.

ROS production, inflammation and apoptosis

Hyperlipidaemia is closely associated with ROS production. Previous studies have shown that ROS are produced when liver cells are exposed to free fatty acids [34]. The production of ROS has been proven to be an important cause of atherosclerosis [35]. In addition, inflammatory molecules and apoptosis-related proteins, such as IL-6, TNF- α , and BAX, were abnormally elevated in patients with hyperlipidaemia [35, 36]. In an animal study, simvastatin administration attenuated brain oxidative stress during experimental sepsis [37]. Consistent with lipid-lowering therapy, the lipid plasmapheresis approach reported here was also associated with a decrease in ROS production, inflammation and apoptosis. It is unknown whether reducing PBMC apoptosis is beneficial in patients with hyperlipidaemia. Previous studies indicated that increases in macrophage and endothelial cell apoptosis were associated with atherosclerosis [38, 39]. Whether the reduction in PBMC apoptosis is beneficial after lipid plasmapheresis remains to be further studied.

Strengths of the study

DFPP treatment for hyperlipidaemia reduced the levels of blood lipids and inflammatory factors. For the first time, it was found that the expression of lipid metabolism-related proteins and endoplasmic reticulum stress-related proteins was reduced, and the levels of ROS was also reduced in PBMCs.

Limitations

There are several potential limitations of our study. First, we did not continuously measure lipid metabolism- and ER stress-related proteins after lipid plasmapheresis, and we could not obtain data concerning when plasma purification treatment should be repeated. Second, there are many lipid-related proteins; some lipid-related proteins were not measured in our study, so it will be necessary to assay all lipid metabolism-related proteins to provide clinicians with complete information about lipid plasmapheresis.

Conclusion

The results of this study demonstrated that DFPP reduced the levels of lipids, lipid-metabolizing proteins, and inflammatory factors in patients with hyperlipidaemia. It also reduced the levels of endoplasmic reticulum stress- and apoptosis-related proteins and ROS in PBMCs. Plasma purification shows promise for the treatment of patients with hyperlipidaemia. Lipid plasmapheresis is also inexpensive, easy to perform, safe and effective.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Yang XH and Hui MJ conceived of and designed the research. All authors conducted experiments and analyzed data. Zhang XM and Deng H wrote the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Zhang XM and Deng H contribute equally to this paper.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All patients provided and signed informed consents. The study was approved by the Ethical Committee of Shanghai Pudong Hospital (Fudan University, Pudong Medical Center, Shanghai, China) and experiments were performed in accordance with the Ethical Committee's guidelines and regulations. ClinicalTrials.gov number was NCT 03491956.

Consent for publication

All authors have approved the final version of the manuscript and have agreed to submit it to this journal.

Competing interests

The authors declare no conflict of interest with respect to the publication of this article.

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Figures

Figure 1

Effects of DFPP on expressions of lipid metabolic proteins The protein expression of LDLR (B), CD36 (C), PCSK9 (D) in PBMCs were detected by Western blot analyses. Pre-DFPP: patients before DFPP. Post-DFPP: patients post DFPP. Values were means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. Pre-DFPP. N=5

Figure 2

DFPP affects ER stress-related proteins ER stress-related proteins [GRP78 (B), ATF4 (C), CHOP (D), p-EIF2α (E)] in PBMCs were detected by Western blot analyses. Values were means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. Pre-DFPP. N=5

Figure 3

Effects of lipid plasmapheresis on expressions of apoptosis-related proteins Apoptosis-related proteins [Bcl-2 (B) BAX (C) Caspase-3 (D)] in PBMCs were examined by Western blotting analyses. Values were means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. Pre-DFPP. N=5

Figure 4

DFPP reduces ROS level ROS level in PBMCs was examined by flow cytometry. Values were means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ vs. Pre-DFPP. N=5