Heparin Cryoprecipitation Reduces Plasma Levels of Non-Traditional Risk Factors for Atherosclerosis in vitro

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Abstract

Aims: To show that heparin cryoprecipitation (HCP), an in vitro method of plasma purification, reduces the levels of in vivo modified proteins and non-traditional risk factors from plasma of atherosclerotic hemodialysis (HD) patients. Methods: HCP was applied to plasma obtained from HD patients and controls, forming a precipitate – cryogel. Levels of fibrinogen, albumin, CRP, TNF-α, IL-6, advanced oxidation protein products, carbonylated fibrinogen and carbonylated albumin were determined in plasma before and after applying HCP and in the cryogel. Results: Treatment of HD plasma with HCP, beyond the significant reduction of the increased levels of all the above-mentioned molecules, reduced fibrinogen, TNF-α, carbonylated fibrinogen and carbonylated albumin to control levels which were simultaneously found in the cryogel. Conclusions: HCP applied to plasma enables the simultaneous precipitation of modified molecules and circulating non-traditional risk factors for atherosclerosis. This study may serve as a base for the future development of a clinical purification technique.

Key Words
Atherosclerosis · Non-traditional risk factors · Oxidized molecules · Inflammation · Heparin cryoprecipitation of plasma

Atherosclerosis and cardiovascular diseases (CVD), which are associated with inflammation, oxidative stress and endothelial dysfunction, remain the major causes of death in the modern world. Although major progress has been made in understanding and modulating risk factors for atherosclerosis, it is still the primary cause of morbidity and mortality in patients suffering from end-stage renal failure treated with chronic hemodialysis (HD) [1]. Most atherosclerotic patients have multiple cardiovascular risk factors, traditional and non-traditional, which potentiate each other, and are usually treated by the pharmacological approach. The classical, traditional, risk factors are usually disease-orientated like hypertension, dyslipidemia and diabetes mellitus, while the non-traditional risk factors focus on specific molecules usually associated with oxidative stress and inflammation processes [1–3].

In inflammation, blood levels of acute phase proteins and cytokines such as fibrinogen, albumin, C-reactive protein (CRP), tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 are modulated, while in severe oxidative stress molecular modifications such as the addition of carbonyls on plasma proteins occur [1, 4, 5]. Albumin and fibrinogen are major plasma proteins which get modified by oxidative stress. Beyond being irreversible and irreparable, carbonylation of proteins by oxidation [6] can lead to diverse functional consequences: tendency of the
protein to aggregate, to be less soluble and to precipitate [5, 7, 8]. In addition, oxidation of albumin, for example, will reduce drug-binding properties and antioxidant properties and may induce tissue factor activity in culture of human umbilical vein endothelial cells [9]. Oxidized albumin is able to trigger the oxidative burst of human neutrophils [10, 11] while oxidized fibrinogen loses the ability to form a solid clot [5, 12], may induce platelet aggregation in the circulation, and enhance IL-8 secretion from human umbilical vein endothelial cells [13].

This study aimed to specifically reduce the levels of modified molecules, especially those that could serve as non-traditional risk factors for atherosclerosis. This was achieved by using the newly developed heparin cryoprecipitation (HCP) method, which is based on an interaction between heparin and proteins, combining freezing temperatures and centrifugation. This precipitation of modified molecules and risk factors forms a pellet, called cryogel. The interaction between heparin and fibrinogen in cooling temperatures has previously been described by Smith et al. [14].

The different effects of temperature, centrifugation and heparin, comprising the HCP method, were evaluated on in vitro modified/untreated fibrinogen and albumin. The HCP method was also applied to commercial TNF-α. Plasma before and after HCP, and cryogels were obtained from atherosclerotic HD patients and the levels of various inflammatory mediators were compared to healthy controls.

HCP preferentially reduces the levels of oxidized proteins, and almost no native proteins, from plasma of atherosclerotic patients. Simultaneously, precipitation of non-modified circulating risk factors was observed from atherosclerotic plasma.

Material and Methods

Blood Samples

Blood was drawn from 25 patients (age range 48–73) with end-stage renal disease on chronic HD treatment, three times a week for 4 h. All the included patients were clinically diagnosed as suffering from accelerated atherosclerosis. 20 healthy controls (HC), age- and gender-matched, served as the control group. Blood from HD patients was always drawn before the start of a dialysis session. The inclusion of HC subjects in the study was based upon clinical examination with laboratory confirmation. Informed consent was obtained from all the patients and subjects participating in this study according to the protocol approved by the institutional committee in accordance with the Helsinki Declaration.

In vitro Oxidation of Fibrinogen and Albumin

Fibrinogen and albumin (Sigma-Aldrich, St. Louis, Mo., USA) were oxidized in vitro using the metal-catalyzed oxidation system comprised of iron and ascorbate to yield a highly oxidized (carbonylated) protein, according to Michellis et al. [15]. Briefly, fibrinogen oxidation (5.5–6 mg/ml) was carried out for 5 h at 37°C in PBS containing 5 mM ascorbate (Sigma, St. Louis, Mo., USA) and 100 μM FeCl₃ (Merck, Germany), while albumin oxidation (28–42 mg/ml) was carried out for 42 h at 37°C in PBS containing 25 mM ascorbate and 100 μM FeCl₃. The reactions were stopped at 4°C by the addition of ethylenediaminetetraacetic acid (EDTA) at pH 8.0 (Sigma) to a final concentration of 1 mM. The oxidizing reagents were removed by overnight dialysis, at room temperature, against PBS.

Determination of Carbonyls Content on Proteins

Levels of carbonylated fibrinogen and albumin were determined, according to Michellis et al. [15]. Briefly, samples were derivatized with 2,4-dinitrophenylhydrazine and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE): 6% for fibrinogen and 10% for albumin. A fixed amount of the in vitro oxidized fibrinogen/oxidized albumin served as a standard [15]. For each protein studied, two gels were run in parallel, one gel was stained for proteins by Coomassie blue, and the other was transferred to nitrocellulose membrane in transfer buffer (25 mmol/l Tris, 192 mmol/l glycine) for Western blot analysis. The detection of carbonyls was performed with rabbit polyclonal anti-dinitrophenyl serum (Sigma) followed by secondary goat anti-rabbit IgG peroxidase conjugate (Sigma). In order to confirm that the carbonyls detected were on fibrinogen/albumin, several nitrocellulose membranes were stripped in water for 30 min and used again for Western blot analysis of fibrinogen/albumin and to untreated proteins (tubes 1, 2).

Solutions of fibrinogen/albumin (Sigma-Aldrich) and their oxidized forms were divided into four tubes after measuring the protein concentration by absorbance at 280 nm. Two tubes contained heparin (14 IU/ml) and two without heparin served as control for each protein (evaluation of the effect of heparin). The concentrations of in vitro fibrinogen/albumin and in vitro oxidized fibrinogen/albumin were measured and compared to their initial concentrations after: a) freezing (~20°C for 24 h) and thawing.
Table 1. Effect of heparin, temperature and centrifugation on the precipitation of untreated fibrinogen (UT-FIB) and oxidized fibrinogen (FIB-CO)

<table>
<thead>
<tr>
<th></th>
<th>Relative initial concentration of fibrinogen</th>
<th>Effect of freezing and thawing (22°C) on fibrinogen precipitation</th>
<th>Effect of centrifugation (4°C) on fibrinogen precipitation</th>
<th>Total precipitation of fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-FIB</td>
<td>100%</td>
<td>-4 ± 2%</td>
<td>-1 ± 4%</td>
<td>-5 ± 4%</td>
</tr>
<tr>
<td>UT-FIB + heparin</td>
<td>100%</td>
<td>-4 ± 3%</td>
<td>-11 ± 4%</td>
<td>-14 ± 3%</td>
</tr>
<tr>
<td>FIB-CO</td>
<td>100%</td>
<td>-21 ± 6%</td>
<td>-25 ± 10%</td>
<td>-40 ± 10%</td>
</tr>
<tr>
<td>FIB-CO + heparin</td>
<td>100%</td>
<td>-19 ± 7%</td>
<td>-37 ± 17%</td>
<td>-49 ± 16%</td>
</tr>
</tbody>
</table>

Table 2. Effect of heparin, temperature and centrifugation on the precipitation of untreated albumin (UT-ALB) and oxidized albumin (ALB-CO)

<table>
<thead>
<tr>
<th></th>
<th>Relative initial concentration of albumin</th>
<th>Effect of freezing and thawing (22°C) on albumin precipitation</th>
<th>Effect of centrifugation (4°C) on albumin precipitation</th>
<th>Total precipitation of albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-ALB</td>
<td>100%</td>
<td>-5 ± 5%</td>
<td>-1 ± 1%</td>
<td>-6 ± 7%</td>
</tr>
<tr>
<td>UT-ALB + heparin</td>
<td>100%</td>
<td>-7 ± 6%</td>
<td>0 ± 3%</td>
<td>-7 ± 6%</td>
</tr>
<tr>
<td>ALB-CO</td>
<td>100%</td>
<td>-14 ± 15%</td>
<td>-3 ± 9%</td>
<td>-17 ± 11%</td>
</tr>
<tr>
<td>ALB-CO + heparin</td>
<td>100%</td>
<td>-11 ± 14%</td>
<td>-14 ± 7%</td>
<td>-23 ± 13%</td>
</tr>
</tbody>
</table>

(22°C), b) freezing (−20°C for 24 h), thawing (4°C) and centrifugation at 4°C for the evaluation of the combined effect of heparin and centrifugation, c) combined effect of heparin, freezing, thawing and centrifugation. The results are shown in tables 1 and 2. Total precipitation refers to the percentage of precipitation following heparin (14 IU/ml), freezing, thawing and centrifugation (800 g).

To summarize the HCP method: the samples were drawn in the presence of 14 IU/ml of sodium heparin, frozen (−20°C for 24 h) and thawed (4°C), followed by centrifugation (4°C, 800 g) and the immediate removal of the supernatant.

In all studies referring to the use of HCP, the same conditions were applied to all samples.

Effect of HCP on Commercial TNF-α
Purified TNF-α (Sigma-Aldrich) was dissolved in PBS and used for in vitro studies. The levels of purified TNF-α were determined by enzyme-linked immunosorbent assay (Bender MedSystems GmbH, Vienna, Austria) before and after application of the HCP method. Commercial TNF-α was prepared in tubes containing 14 IU/ml of sodium heparin (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) (Before HCP), frozen for 24 h (−20°C), then thawed to 4°C and centrifuged again (800 g) at 4°C, followed by the immediate removal of the supernatant (After HCP).

Application of HCP to Plasma
Blood was drawn into tubes containing 14 IU/ml of sodium heparin (Vacuette®, Greiner Bio-One GmbH), centrifuged at 800 g to obtain cell-free plasma (Plasma before). This plasma was frozen for 24 h, then thawed at 4°C and centrifuged again (800 g) at 4°C, followed by the immediate removal of the supernatant (Plasma after). This procedure resulted in a pellet referred by us as cryogel. The cryogel was insoluble in cold temperatures but mostly soluble at 37°C.

Determination of Biochemical and Inflammatory Parameters
Fibrinogen and albumin levels in plasma and cryogel of HD patients and HC subjects were determined using the Cobas Mira analyzer (Roche Diagnostics, Basel, Switzerland) using the K-assay kit (Kamiya Biomedical Co., Seattle, Wash., USA) for fibrinogen and using a colorimetric assay for albumin (Sentinal Diagnostics, Milan, Italy). Plasma and cryogel levels of IL-6, CRP and TNF-α in HD patients and HC were determined using enzyme-linked immunosorbent assay by Bender MedSystems GmbH.

Determination of Advanced Oxidation Protein Products
Advanced oxidation protein products (AOPP) serve as general markers for the evaluation of protein oxidation. AOPP levels in plasma and cryogel of HD patients and HC were evaluated. Determination of AOPP is based on spectrophotometric detection [16]. Plasma samples were diluted 1:5 in PBS; pure acetic acid (99%; Sigma) was added to all tubes and the absorbance at 340 nm was measured immediately. A standard calibration curve was performed using Chloramine-T solution (Sigma, The Netherlands), 0–100 μmol/l, followed by the addition of 1.16 mol/l potassium iodide (Sigma). PBS served as a blank.

Protein Phenotyping of Cryogel
Analysis of the protein contents of the cryogel by sequencing and identification was carried out by mass spectrometry, in the
Smoler Proteomic Center, Department of Biology, Israel Institute of Technology, Technion, Haifa, Israel.

Statistical Analysis
Data are expressed as means ± SD. Values were compared by Wilcoxon signed ranks test. A p value < 0.05 was considered statistically significant.

Results

Effect of Temperature, Centrifugation and Heparin on in vitro Protein Precipitation
The separate effects of heparin, temperature and centrifugation on the precipitation of commercial oxidized fibrinogen/albumin vs. control, untreated fibrinogen/albumin were evaluated. The results are shown in table 1 for fibrinogen and table 2 for albumin.

Untreated fibrinogen (UT-FIB), with or without heparin, followed by freezing and thawing (22°C), resulted in the precipitation of a small percentage of fibrinogen (4%), indicating that heparin, freezing and thawing had very little effect on the precipitation of native UT-FIB. Centrifugation of these tubes (4°C) resulted in additional precipitation of the UT-FIB, but to a much greater extent in the presence of heparin (11 vs. 1%).

Freezing and thawing of the in vitro oxidized fibrinogen (FIB-CO), with and without heparin, resulted in about 20% precipitation of the protein. The addition of centrifugation resulted in further precipitation of the protein, which was augmented by the addition of heparin (49 vs. 40%). Both FIB-CO and UT-FIB precipitated more in the presence of heparin, although modified fibrinogen precipitated more than the native protein (table 1, n = 4).

The conditions (heparin 14 IU, freezing –20°C, thawing 4°C, centrifugation 800 g, 4°C) where we succeeded in precipitating maximum FIB-CO were used in all the following experiments and are referred to hereafter as HCP. The effect of HCP on the precipitation of commercial fibrinogen is also shown in figure 1a.

The in vitro FIB-CO was precipitated from an average of 484 ± 98 mg/dl before application of HCP to 247 ± 32 mg/dl after the application of HCP (49% decrease), while UT-FIB was precipitated from 512 ± 127 to 439 ± 93 mg/dl (14% decrease) (fig. 1a).

The levels of carbonyls in the UT-FIB and in vitro FIB-CO are depicted in figure 1b. Higher levels of carbonyls per fibrinogen molecule were expressed on in vitro FIB-CO compared to UT-FIB, 3.21 ± 1.45 vs. 0.54 ± 0.47 nmol carbonyl/mg fibrinogen, respectively. HCP significantly reduced the levels of the highly carbonylated fibrinogen molecules, from 3.21 ± 1.45 to 2.30 ± 1.26 nmol carbonyl/mg (28% reduction) while in UT-FIB the lower carbonyl levels were non-significantly reduced from carbonyl levels of 0.54 ± 0.47 to 0.47 ± 0.28 nmol carbonyl/mg; the higher the carbonylation, the greater the precipitation.
Commercial untreated albumin (UT-ALB), with or without heparin, followed by freezing and thawing (22°C), resulted in the precipitation of a small percentage of albumin (about 5–7%), indicating that heparin, freezing and thawing had very little effect on the precipitation of native UT-ALB.

Cold temperatures and centrifugation of these samples did not add to the precipitation of native albumin (table 2, n = 3).

Freezing and thawing of the in vitro oxidized albumin (ALB-CO), with and without heparin, resulted in about 11–14% precipitation of the protein. The addition of cold temperatures and centrifugation resulted in an additional precipitation of the protein, which was augmented by the presence of heparin (23 vs. 17%). ALB-CO precipitated to a greater extent in the presence of heparin (table 2).

The particular effect of HCP on the precipitation of commercial albumin is also shown in figure 2a.

ALB-CO was precipitated from 3.41 ± 0.7 to 2.58 ± 0.3 g/dl (23% decrease) while UT-ALB was precipitated...
The levels of carbonyls on untreated and oxidized albumin are depicted in figure 2b. ALB-CO expressed higher levels of carbonyls per albumin molecule compared to UT-ALB and had a higher carbonyl content of 8.82 ± 5.05 vs. 1.65 ± 0.46 nmol carbonyl/mg albumin, respectively. HCP reduced significantly highly carbonylated albumin molecules from 8.82 ± 5.05 to 7.40 ± 3.54 nmol carbonyl/mg (16% decrease) albumin while in UT-ALB the carbonyl levels were not reduced (1.65 ± 0.46 to 1.57 ± 0.62 nmol carbonyl/mg albumin) following HCP; the higher the carbonylation, the greater precipitation.

The difference of the carbonyl content on in vitro FIB-CO vs. ALB-CO (8.82 ± 5.05 nmol carbonyl/mg for albumin vs. 3.21 ± 1.45 for fibrinogen) is a result of the different oxidation protocol. Albumin was oxidized for a much longer period and with higher ascorbate contents than fibrinogen (see Material and Methods).

**Effect of HCP on HD and HC Plasma**

Plasma from atherosclerotic patients on chronic HD treatment and from healthy subjects was evaluated before and after the application of HCP. The effect of HCP on inflammatory parameters and modified molecules in HD and HC plasma is stated in table 3. Significantly elevated levels of fibrinogen, CRP, IL-6 and oxidized molecules such as advanced oxidative protein products, carbonylated fibrinogen and carbonylated albumin and significantly lower levels of albumin characterized the HD plasma. Following treatment by HCP, the levels of fibrinogen, albumin, CRP, TNF-α, IL-6, AOPP, carbonylated fibrinogen, and carbonylated albumin significantly decreased in HD plasma. It must be emphasized that the pathologically high levels of fibrinogen and TNF-α in HD plasma were corrected to the normal healthy plasma level. In contrast, the only parameter that decreased significantly in HC treated by HCP was fibrinogen, which did not precipitate below the normal range (200–400 mg/dl). In both groups, the levels of non-carbonylated albumin were hardly affected.

**Effect of HCP on the in vitro Precipitation of TNF-α**

TNF-α is a main inflammatory cytokine involved in inflammatory states. Since high levels of TNF-α were found in HD plasma (table 3), we studied the effect of HCP on the non-traditional risk factor TNF-α precipitation in vitro. TNF-α concentrations before and after application of the HCP method were measured. HCP significantly precipitated commercial TNF-α levels from an average of 2.79 ± 0.87 to 2.03 ± 0.45 pg/ml (~27%).

**Cryogel Composition**

**SDS-PAGE Analysis of Cryogel.** A representative picture of three different concentrations of HD cryogel as analyzed by SDS-PAGE is shown in figure 3. Cryogel was precipitated from HD plasma, separated on SDS-PAGE (8%), stained by Coomassie blue and analyzed. The HD cryogel contained fibrinogen (340 kDa), immunoglobulins (150 kDa), transferrin (76 kDa), and albumin (66 kDa), in addition to other unidentified proteins (fig. 3).

**Mass Spectrometry Analysis of Cryogel.** The main proteins identified in the HD cryogel after analysis by mass spectrometry are depicted in table 4. It can be seen that the cryogel contains fibrinogen, haptoglobin, immunoglobulins, transferrin, and albumin. This analysis is semiquantitative, and the detected proteins shown in table 4 are in a decreasing order as to their relative amounts in the cryogel. This analysis supported the results obtained by the SDS-PAGE (fig. 3).

**Characterization of Fibrinogen/Carbonylated Fibrinogen in Plasma and Cryogel.** Plasma and cryogel of HD and HC were separated on SDS-PAGE (6%) and analyzed by Western blot analysis for fibrinogen and carbonylated fibrinogen (fig. 4a, b). HD plasma contained higher levels of fibrinogen, its degradation products, aggregates and carbonylated forms than form HC plasma. The HCP method reduced all forms of fibrinogen, which are abundant in the cryogel (fig. 4a, b). When comparing cryogel
from HC and HD it is evident that the amount of fibrinogen, its degradation products, aggregates and its carbonylated forms are precipitated to a greater extent in HD than in HC plasma (fig. 4a, b). The quantified fibrinogen and carbonylated fibrinogen levels in plasma are shown in table 3 and in cryogel in table 5. It has to be noted that the oxidation causes higher molecular weight aggregates in HD than in HC (fig. 4b).

Characterization of Albumin/Carbonylated Albumin in Plasma and Cryogel. Plasma and cryogel of HD and HC were separated on SDS-PAGE (10%) and analyzed by Western blot analysis for albumin and carbonylated albumin (fig. 5a, b). HD plasma contained higher levels of carbonylated albumin than HC plasma, which was reduced by the HCP method (fig. 5b). Cryogel from HD showed significantly higher amounts of carbonylated albumin than HC cryogel (fig. 5b). The quantified levels of...
albumin and carbonylated albumin are shown in plasma (table 3) and in cryogel (table 5).

A summary of cryogel contents is depicted in table 5. HD cryogel contained significantly higher levels of fibrinogen, CRP, TNF-α, IL-6, AOPP, carbonylated fibrinogen and carbonylated albumin, while albumin levels were similar in both HD and HC cryogels.

### Discussion

HCP, a unique molecular precipitating method, was developed in this study. This method combines the use of heparin, freezing temperatures and centrifugation to preferentially precipitate a cluster of risk factors from atherosclerotic patients’ blood (in vitro).

During the development of the HCP method, we tried to find the optimal conditions to precipitate mainly modified molecules and cardiovascular risk factors while trying to avoid precipitation of native molecules. Preliminary studies evaluating the effect of various concentrations of heparin on precipitation level were carried out. These studies indicated that 14 IU/ml of sodium heparin is the lowest level of heparin required for application of the HCP method (data not shown). Heparin, a highly charged polyanion, is capable of forming soluble and insoluble complexes with proteins, which upon freezing and the application of centrifugal force will accelerate their precipitation [17, 18]. The precipitate or the cryogel is insoluble in cold temperatures but mostly soluble at 37°C, enabling its removal by centrifugation at lower temperatures. We show that heparin complexes of native proteins hardly precipitate, while complexes with these in vitro oxidized proteins (oxidized albumin and oxidized fibrinogen) precipitate better by freezing, thawing and centrifugation.

Plasma of atherosclerotic patients on chronic HD contains high levels of modified molecules and high levels of circulating non-traditional risk factors for atherosclerosis such as AOPP, carbonylated fibrinogen, carbonylated albumin, fibrinogen, CRP, IL-6 and TNF-α. All these parameters, together with the low levels of albumin, reflect a state of systemic oxidation and inflammation in these patients [1–3, 11, 13, 15, 19, 20].

When the HCP method was applied to plasma of HD patients, acute phase proteins and cytokines such as fibrinogen, CRP, IL-6, TNF-α and modified molecules such as AOPP, carbonylated fibrinogen and carbonylated albumin precipitated, while hardly any precipitation was observed in HC plasma. HD plasma contained significantly higher levels of AOPP, CRP, IL-6 and TNF-α levels than HC, which were significantly reduced by the HCP method.

It is interesting to note that in our healthy subjects, fibrinogen levels that were in the upper quartile of the normal range precipitated by the HCP to the lower normal levels. The risk of precipitating native molecules by HCP is one of the hazards of this method. Nevertheless, the described HCP method precipitated threefold more of the oxidized proteins compared to the native ones, with a minimal effect on the native ones. As other studies have shown that fibrinogen and its carbonylated form are in-

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Table 4. Mass spectrometry analysis of the HD cryogel

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular weight</th>
<th>KDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>140–900</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>86–1,000</td>
<td></td>
</tr>
<tr>
<td>β-Globin</td>
<td>~16</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>Apoprotein A</td>
<td>300–800</td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>~130</td>
<td></td>
</tr>
<tr>
<td>Apoprotein C</td>
<td>~10</td>
<td></td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>&gt;220</td>
<td></td>
</tr>
<tr>
<td>Complement components (C1q, C3, C4)</td>
<td>190–410</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SD of the following sample size: fibrinogen and albumin, n = 15 for HD, n = 10 for HC; CRP, TNF-α, and IL-6, n = 8 for HD, n = 4 for HC; AOPP, FIB-CO and ALB-CO, n = 10 for HD, n = 5 for HC. FIB-CO = Carbonylated fibrinogen; ALB-CO = carbonylated albumin.

*p < 0.05 vs. HC cryogel.

Table 5. Cryogel contents: acute phase proteins, cytokines and modified molecules

<table>
<thead>
<tr>
<th></th>
<th>HD cryogel</th>
<th>HC cryogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen, mg/dl</td>
<td>239±114*</td>
<td>153±66</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>0.38±0.14</td>
<td>0.4±0.12</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>1.0±1.0*</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>0.84±0.58</td>
<td>0.78±0.33</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>0.96±0.67*</td>
<td>0.12±0.12</td>
</tr>
<tr>
<td>AOPP, μM</td>
<td>26.6±10*</td>
<td>7.7±4.8</td>
</tr>
<tr>
<td>FIB-CO, nmol Carb/mg protein</td>
<td>7.19±4.6*</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>ALB-CO, nmol Carb/mg protein</td>
<td>6.8±2.45*</td>
<td>2.37±1.23</td>
</tr>
</tbody>
</table>

Results are mean ± SD of the following sample size: fibrinogen and albumin, n = 15 for HD, n = 10 for HC; CRP, TNF-α, and IL-6, n = 8 for HD, n = 4 for HC; AOPP, FIB-CO and ALB-CO, n = 10 for HD, n = 5 for HC. FIB-CO = Carbonylated fibrinogen; ALB-CO = carbonylated albumin.

*p < 0.05 vs. HC cryogel.
chronic HD patients, we imply that significant reduc-
tion in their levels from HD plasma may be beneficial in
attenuation of the atherosclerotic process and cardiovas-
cular morbidity in these patients [3, 5, 13, 21, 22].

Hypoalbuminemia, frequently observed in HD pa-
tients, is a measure of malnutrition, acute phase inflam-
mation, accelerated atherosclerosis (MIA syndrome), and
a strong predictor of cardiovascular morbidity and morta-
rity [23–26]. Himmelfarb and McMonagle [19] and
Mera et al. [11] have reported that HD patients on chronic
HD have lower albumin and higher carbonylated albu-
mun levels than HC. We show that the HCP method is
efficient in precipitating particularly the carbonylated
albumin, without affecting the levels of the unmodified
molecules both in HD patients and HC subjects. This is
especially important in view of accumulating data show-
ing that decreased albumin levels predict mortality in all
populations, and are a powerful predictor of CVD in
chronic HD patients [27]. Oxidized albumin, in addition
to being a reliable marker for oxidative stress in HD pa-
tients [11], triggers an oxidative burst of human neutro-
phils and as such serves as a prooxidant too [9–11]. There-
by, the reduction in oxidized albumin levels from HD
blood by HCP will contribute to the attenuation of the
systemic oxidative stress in these atherosclerotic patients.
A lack of interaction between the anionic albumin with
the polyanionic heparin may explain the minor effect of
heparin on the precipitation of the native albumin, nev-
evertheless the HCP method precipitates better the ox-
dized form of albumin.

Our findings support the study by Himmelfarb and
McMonagle [19] which demonstrated that albumin is the
major plasma target of excess oxidation in HD patients.
Albumin can accumulate oxidative modifications possi-
dibly due to its long turnover [28] in plasma (15–19 days);
this, together with the high albumin concentration in
plasma, compose albumin as the major plasma antioxi-
dant protein.

AOPP is a surrogate marker of oxidative stress and
serves as an indicator for protein oxidation [29]. AOPP
also mediates inflammation and triggers oxidative reac-
tions of neutrophils, monocytes and T lymphocytes, which
contribute to inflammation and to the impairment of the
immune system found in atherosclerosis [30]. The reduc-
tion in AOPP levels by HCP from HD plasma, beyond con-
firming our hypothesis that HCP is an effective method in
the reduction of modified molecules, may also be benefi-
cial in reducing the inflammatory state in HD patients.

The reduction of CRP by HCP is also of biological im-
portance. Beyond being a reliable marker for inflamma-
tion and a strong predictor of future cardiovascular
events [31], CRP is suggested as a mediator of the ather-
sclerotic process [32]. CRP production, which is predomi-
nantly under the control of IL-6, is involved in the induct-
on of adhesion molecule expression in human endothe-

dlial cells, is able to activate the complement system [32],
and may play a direct role in promoting the inflamma-
tory component of atherosclerosis [31–33].

IL-6 and TNF-α are central cytokines involved in the
proinflammatory process. IL-6 is suggested as a predic-
tor of future heart disease and is the only cytokine that
can stimulate the synthesis of all acute phase proteins in-
volved in the inflammatory response [21].

TNF-α is regarded as the master regulator of the cy-
tokine cascade. TNF-α induces thrombotic and inflam-
atory reactions in endothelial cells, and also induces
production of IL-6 by these cells [34]. We suggest that the
highly significant reduction in TNF-α from HD plasma
may result from its binding to heparin [35], forming a
complex that is then precipitated by the HCP method.

By reducing the levels of all the mentioned cytokines
and inflammatory mediators, a deceleration and retarda-
tion of the viscous cycle of oxidative-stress-inducing in-
flammation will be achieved, finally attenuating the ath-
erosclerotic process.

As a mirror image, the HD cryogel contained signifi-
cantly higher levels of fibrinogen, CRP, IL-6, AOPP, car-
bonylated fibrinogen, and carbonylated albumin com-
pared to HC cryogel, confirming the enhanced precipita-
tion of these inflammatory mediators and modified
molecules from HD plasma.

Treatment of atherosclerosis is principally by the tra-
ditional pharmacological approach which deals mainly
with one risk factor at a time, and is only partially suc-
cessful in the reduction of CVD morbidity/mortality.

The heparin-mediated extracorporeal low-density li-
oprotein precipitation procedure (HELP apheresis) is
the main non-traditional technique used for selective re-
moval of plasma low density lipoproteins in the indica-
tion of familial hypercholesterolemia unresponsive to
medication. This procedure is based on application of
high levels of heparin (100 IU/ml) together with the lower-
ing of plasma pH values [36].

HELP apheresis manages to reduce levels of proath-
erogenic lipoproteins and of several circulating proin-
flammatory markers as well [36]. Although HCP uses
lower levels of heparin and does not require the lowering
of the pH values, our results are in agreement with results
presented by Wang et al. [37], showing reduction in fi-
brinogen and CRP levels. It has to be emphasized that
HCP did not reduce fibrinogen levels below normal levels as in the data presented by Wang et al. [37]. Many atherosclerotic patients have normal levels of atherogenic lipoproteins [38], suggesting that they are not always the major risk factor of atherosclerosis. These patients do not qualify for treatment by HELP apheresis.

In order to effectively attenuate the atherosclerotic process, there is a genuine need for a new therapeutic approach, such as HCP, which can simultaneously reduce multiple risk factors, independent of lipoprotein levels.

This study evaluated the effect of HCP on the in vitro precipitation of modified molecules and circulating non-traditional risk factors for atherosclerosis in plasma removed from HD patients. We believe that the data presented in this study may serve as a basis for future studies focusing on the development of an applicable in vivo clinical purification technique. Additionally, evaluation of the effect of HCP in vitro on plasma removed from other groups of atherosclerotic patients (such as patients with dyslipidemia/hypercholesterolemia) has to be carried out. The HCP method may be developed to complement the pharmacological treatments designed to reduce levels of circulating non-traditional risk factors for atherosclerosis and CVDs, in achieving attenuation in the propagation of atherosclerosis.

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References


