Heparins and blood polymorphonuclear stimulation in haemodialysis: an expansion of the biocompatibility concept

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Abstract

Background. At the concentrations used in haemodialysis and in a dose-dependent way, unfractionated heparin (UFH) and, to a lesser degree, a low-molecular-weight heparin (LMWH) stimulate polymorphonuclear cells (PMN) in vitro, and could act in synergy with the stimulatory effect of dialysis membranes in vivo. To examine this hypothesis, we studied the effects of different heparin types and regimens on blood PMNs during haemodialysis sessions.

Methods. Ten haemodialysed patients were studied during regular dialysis sessions on a cellulose triacetate membrane (CT 110 G; 1.10 m²; Baxter), with four different random heparin protocols: one high-UFH regimen (HHR) at 90 IU/kg body-weight (b.w.) and one low-UFH regimen (LHR) at 50 IU/kg b.w., and with a LMWH (nadroparin calcium) at 85 (HHR) or 45 (LHR) IU/kg b.w. Blood granulocytes, platelet counts, and plasma granulocyte degranulation products (elastase, lactoferrin) were measured serially during 4 h dialysis sessions.

Results. After 10 min, the reduction in PMNs with UFH was 29.5% for HHR (P<0.01) and 28.5% for LHR (P<0.01), and only 16.8 and 18.6% with LMWH (NS), significantly higher for HHR with UFH than with LMWH (P<0.01). At 60 min, the elastase increase with HHR was greater, 61% with UFH (P<0.01) and 37.8% with LMWH (P<0.01), significantly higher than LHR for UFH (P<0.05) or LMWH (P<0.05). The overall decrease in platelets (with LMWH P<0.01) and the overall increase in lactoferrin (P<0.001) were not different between heparinization procedures.

Conclusion. Under a conventional heparin regimen, the PMN variation during the course of the dialysis session suggests a more biocompatible effect of LMWH over UFH. In addition, the variation of elastase favours the lower dose, whatever the type of heparin. Heparin type and dose should therefore be considered in studies addressing biocompatibility in haemodialysis: a low dose of LMWH may be viewed as a better biocompatible treatment with regard to leukocyte stimulation.

Keywords: biocompatibility; elastase; haemodialysis; heparins; lactoferrin; polymorphonuclear cells

Introduction

The concept of biocompatibility in haemodialysis has gradually expanded in recent years [1], and usually refers to the entire extracorporeal circuit, the dialysis bath, and the sterilizing agents. Unfractionated heparin (UFH), used at an annual dose of 800 000 to 1 500 000 IU and for 10–20 years, has been only recently recognized as a potential factor affecting biocompatibility [2,3], and its biological effects have been rarely considered in vitro [4,5], ex vivo [6], or in vivo [7–10].

Various polymorphonuclear neutrophil (PMN) end-functions can be modulated by heparins. At concentrations (anti-Xa activities) higher than those used in haemodialysis, a more PMN-inhibiting effect of UFH over a low-molecular-weight heparin (LMWH) has been reported in vitro. This action is considered to be a dose-dependent effect [11–16] and linked to the size, charge, and degree of sulphation [11,13,16] which favours LMWH. Conversely, at lower concentrations, such as those used in haemodialysis, the PMN-stimulating action of heparins has been poorly investigated as regards adhesion [12], aggregation [14,17], degranulation [15,17], and oxidative metabolism [15]. This stimulatory effect is confirmed in vivo by intravenous (i.v.) UFH which can elicit leukopenia in rabbits [17]. However, the heparin dose-dependent stimulatory action has only been demonstrated in vitro for UFH [12,14,15,17], while a LMWH has been shown to be less stimulatory and to weakly inhibit superoxide ion production [15]. This observation with normal human...
PMNs has been confirmed with end-stage renal disease (ESRD) patients' PMNs [15]. Therefore the UFH PMN stimulatory effect could contribute to the long-term complications of maintenance dialysis [2,3] such as atherogenesis [18], and amyloidosis for which the role of glycosaminoglycans has been proposed [19]. In contrast, a LMWH could have a less stimulatory action and thus a potential protective effect, as suggested on bone metabolism in animals [20] and lipid profile in ESRD patients [21].

In order to test this hypothesis in vivo, we conducted a random study to determine the effect of two types of heparin, and for each one the effect of two usual doses used on chronic haemodialysis, on the activation of systemic leukocytes of ESRD patients during the course of a haemodialysis session.

Subjects and methods

Design

Each patient underwent four studies, two haemodialysis sessions with an UFH and two sessions with a LMWH. For each type of heparin, one session was conducted using a high-heparin regimen (HHR), and the other using a low-heparin regimen (LHR). The order of inclusion was allocated according to a computerized randomization model and a 2-week period separated each study. With the exception of the heparinization procedures used, the other technical dialysis conditions were identical for each patient, and used for at least 1 month prior to the study. The University and Hospitals Ethics Review Committee gave its approval to the procedures.

Patients

Ten patients were included after giving informed consent: six men, four women, mean age 57.8 years (range 33 to 72), mean duration treatment on haemodialysis 7.9 years (range 2.5–14) and for at least 1 year with a high-permeability dialysis membrane: polyacrylonitrile (AN69) (Hospal, Meyzieu, France) (four patients), polysulphone (Fresenius, Bad Homburg, Germany) (two patients), polymethylmethacrylate (Toray, Tokyo, Japan) (two patients), and cellulose triacetate (Baxter, IL, USA) (two patients). The causes of renal failure were primary glomerulonephritis (4), interstitial nephritis or uropathy (3), renal vascular disease (2), and polycystic kidney disease (1). All patients were anuric, in a stable clinical condition, and none of them had any other disease or was taking any medication known to affect leukocyte function.

Dialysis and heparinization protocol

The dialysis sessions were conducted over 4 h with a bicarbonate bath prepared with ultrapurified water (<2 colony-forming units (c.f.u.)/100 ml, endotoxins <0.06 EU/ml). The calcium concentration in the bath was 1.50 mmol/l. The ultrafiltration required by the patient was constant throughout the session. The high-permeability capillary-type dialysis membrane was made of cellulose triacetate (CT 110 G; 1.10 m²; Baxter, IL, USA), and blood lines were polyvinylchloride. The entire extracorporeal circuit was sterilized by gamma radiation, and was rinsed before the dialysis session with a 2000 ml NaCl isotonic solution, without heparin. The blood flow rate was 200 ml/min during the entire session.

Anticoagulation by UFH was carried out using the same batch of sodium heparin, without additive or preservative (333 IU/ml; Roche, Neuilly sur Seine, France). For HHR, an i.v. bolus of 30 IU/kg b.w. was followed by a constant infusion of 15 IU/kg b.w./h (total dose 90 IU/kg b.w., range 4100–7400 IU). For LHR, a constant infusion of 12.5 IU/kg b.w. was conducted (total dose of 50 IU/kg b.w., range 2300–4100 IU), without a starting bolus. The infusion was continued up to the end of the dialysis session. For LMWH, the same batch of nadroparin calcium, without any additive or preservative (3075 IU/ml; Sanofi-Synthelabo, Le Plessis Robinson, France) was used. A single i.v. bolus was performed at the start of dialysis: 85 IU/kg b.w. for HHR (range 3800–6700 IU), and 45 IU/kg b.w. for LHR (range 2100–3700 IU), without further administration.

Blood samples

The samples were taken from the arterial needle, before the extracorporeal circuit was started and before injection of the anticoagulant (0 min), then from the outlet side, i.e. from the venous side. Blood was drawn on sodium citrate for heparin, on tripotassium EDTA for PMN degranulation markers and cell counts, and on lithium heparin for total proteins. The samples were chilled on ice and sent for analysis within 1 h.

Plasma analyses

Plasma heparin level was studied at 0, 10, 60, 120 and 240 min. The measurement of heparin activity was conducted as anti-Xa (aXa) inactivation using chromogenic substrate (S. 2337) and bovine factor X (Chromogenix, Milano, Italy) [22]. Different calibrations were used for UFH (UFH IU) and LMWH (LMWH IU). The measurements were all performed using a Corning spectrophotometer.

Elastase and lactoferrin were measured in duplicate at 0, 10, 60, 120 and 240 min as markers of PMN degranulation. The measurement methods have been reported in detail elsewhere [15,23]. Briefly, elastase in a complex with α1-proteinase inhibitor, and lactoferrin were measured by highly sensitive enzyme-linked immunoassay, using a kit obtained from Merck (Darmstadt, Germany) for elastase and using antihuman lactoferrin rabbit IgG (Dako, Glostrup, Denmark) for lactoferrin. Sensitivity and reproducibility were 100 ng/ml and 97.9% for elastase, and 5 ng/ml and 96% for lactoferrin respectively.

Total proteins were measured using the biuret method (Hitashi 737). The haemconcentration in the course of the dialysis session was estimated by using the quotient of protein concentration of the sample and of baseline protein concentration to correct the plasma values and cell counts.

Blood cell counts

PMNs and platelets were measured in duplicate at 0, 10, 60, 120 and 240 min using an automatic counter, ABX Minos.

Statistics

The data corrected for haemconcentration were obtained from repeated measures design with three within-subjects...
factors: two factors at two levels, heparin type and heparin regimen, and a time factor at five levels not equally spaced. Therefore, multivariate analysis of variance (MANOVA) with a difference contrast and repeated measurement design, was used to characterize the patterns of change of dependent variables over time factor [24]. This method allows the analysis, in a global way, of the overall variation of dependent variables during the dialysis session. If necessary, a reference time-point was also chosen, at 10 min for PMN counts and at 60 min for plasma elastase. With each heparinization condition, a heparin regimen effect was studied by comparing LHR and HHR for the same type of heparin, and a heparin type effect was studied by comparing LMWH and UFH for the same regimen. The statistical significance of linear correlations was interpreted by the Fisher’s transformation test.

The homogeneity of values for each variable was verified at the start of the session (0 min) in the four conditions. If this homogeneity hypothesis was rejected and to avoid this bias, the analysis of absolute values during the dialysis session was replaced by that of relative values in comparison with the values at 0 min.

All calculations were conducted using the SPSS program MANOVA (release 6.1 of SPSS Windows, IL, USA). The level of significance was chosen at 0.05. But due to the small number of subjects studied (n=10), we considered that a level of 0.10 could also be indicated to bring down type II error and to suggest a significant trend [25].

Results

Ultrafiltration and haemoconcentration

The mean ultrafiltration during the session was 3001 ± 207 ml (range 2778 ± 227 to 3167 ± 195 ml for the four conditions studied) (mean ± SEM). The mean haemoconcentration at the end of the session reached 28.9 ± 6.0% (range 25.9 ± 4.8 to 30.1 ± 6.1% for the four studies). There were no statistical significant difference between the four situations tested.

Degree of heparinization (Figure 1)

The overall variation of heparin activity during the session was studied from 10 min by aXa activity. For HHR, the mean value of the heparin activity was stable, being below 0.46 IU/ml with UFH, whereas with LMWH the aXa activity reached 1.25 IU/ml at 10 min and then steadily decreased to 0.56 IU/ml at the end of the session. For LHR, aXa activity reached 0.22 IU/ml at the end of the session with UFH, whereas with LMWH it rose to 0.72 IU/ml at 10 min and then decreased to 0.29 IU/ml at the end of the session. For LHR, mean heparin concentrations were significantly lower as compared with HHR, with UFH (P<0.001) as well as LMWH (P<0.05).

Blood cell counts

The variation of cell counts in the course of the session was studied from baseline (0 min). There was no difference between values at baseline.

The overall variation in PMNs (Figure 2) was highly significant with UFH (P<0.001), whereas we observed a tendency with LMWH (P=0.09). At 10 min with UFH, the PMNs decrease was significant, reaching 29.5% for HHR (P<0.01) and 28.6% for LHR (P<0.01). With LMWH, this decrease was 16.8 and 18.6% respectively (NS). An effect of heparin type only existed statistically at 10 min for HHR, with a greater decrease with UFH than with LMWH (P<0.01).

The steadily decrease in platelets exceeded 10% at 240 min (results not shown). The overall variation in platelet counts was significant for LMWH (P<0.01) and only with a tendency for UFH (P=0.08). No regimen or heparin type effect was observed.

None of the overall cell variations during the dialysis session, and PMN decrease at 10 min, was related to the heparin activity at 10 min (aXa, UFH IU or LMWH IU). In addition, the PMN counts at other time-points of the session were not related to the heparin activity.

Plasma markers of PMN degranulation

Variation of variables during the session were analysed from baseline (0 min).

The four baseline levels of plasma elastase were not
Fig. 2. Variation of PMN count during the dialysis session (PMN, polymorphonuclear leukocytes) with an unfractionated heparin (UFH) or a low-molecular-weight heparin (LMWH), and with a high-heparin regimen (HHR, continuous line) or a low-heparin regimen (LHR, dashed line); mean ± SEM. Overall variation from baseline (0 min). a, P = 0.09, d, P < 0.001. No significant global difference between the heparin types or between the heparin regimens. Variation at time-point 10 min from baseline. & , P < 0.01. Difference between the heparin types with HHR, LMWH vs UFH, P < 0.01.

statistically different. In an in vitro assay we found that elastase level was not affected by UFH or by LMWH in a range of aXa activity up to 1 IU/ml, and was reduced by less than 10% up to 2.1 IU/ml, without difference between the heparin types (data not shown). The overall variation in elastase (Figure 3) was significant during the four studies. From baseline to 60 min, a significant increase in elastase was observed with HHR, reaching 61% with UFH (P < 0.01) and 37.8% with LMWH (P < 0.01). Until 60 min, there was no significant heparin type effect, but a regimen effect was significant with UFH (P < 0.05) as well as with LMWH (P < 0.05). Variations up to this time point were not correlated with the heparin activity at 10 min.

During UFH, the mean lactoferrin value at baseline was higher for HHR than for LHR (P < 0.05). Therefore analysis was conducted with relative values compared to the baseline values. The overall increase in lactoferrin mostly reached a plateau profile and was significant for all procedures (P < 0.001) (Figure 4). No heparin type or regimen effect was observed.

Discussion

We report for the first time the stimulatory effect of two types and doses of heparin on blood PMNs during standard haemodialysis in ESRD patients.

Fig. 3. Variation of plasma elastase during the dialysis session with an unfractionated heparin (UFH) or a low-molecular-weight heparin (LMWH), and with a high-heparin regimen (HHR, continuous line) or a low-heparin regimen (LHR, dashed line); mean ± SEM. Overall variation from baseline (0 min). b, P < 0.05; c, P < 0.01; d, P < 0.001. No significant global difference between the heparin regimens or between the heparin types. Variation at time-point 60 min from baseline. & , P < 0.01. Difference between the heparin regimens with UFH and with LMWH: LHR vs HHR, P < 0.05.

Unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH, nadroparin calcium) were tested at dosages corresponding to those usually administered in chronic haemodialysis (high-heparin regimen, HHR) and at doses adapted to minimum administration in the context of bleeding risk (low-heparin regimen, LHR) [26–28]. We found that aXa (UFH IU and LMWH IU) for each type of heparin was comparable to those recommended (Figure 1) [26,27]. The extracorporeal circuit characteristics used are considered to meet acceptable biocompatibility standards [1–3], including the use of a cellulose triacetate high-permeability dialysis membrane. This membrane is deemed to be as biocompatible as synthetic high-permeability membranes [29,30]. Blood samples were collected from the outlet side, i.e. on the venous side, instead of the inlet side, because it appears to be more relevant when addressing functional changes of the PMNs [31–33].

We show that the action of heparin on early granulocytopenia (at 10 min) depends on the nature of heparin and on heparin regimen (Figure 2). Our observation in vivo is in agreement with the stimulant action of UFH on PMN adhesion and aggregation described in vitro [12,14,17] and on blood PMN count in vivo in animals [17]. The activity of heparin on PMNs depends on electric charge and length of chain [11,13,16], which...
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may explain the more pronounced effect of UFH on the PMN count as compared to LMWH. In addition, in vitro, this stimulating action depends on heparin concentration (aXa) [12,14,17]. We observed a particular granulocytopenic effect of the high regimen with UFH as compared to LMWH. These findings were not observed by Otte et al. for total leukocyte counts, since these authors only studied various administrations of the same dose of UFH [7]. In the present study, we can rule out a potential pharmacokinetic difference of heparin action on PMNs, since both heparins were used intravenously and granulocytopenia was observed in the early session.

The PMN degranulation with increased plasma elastase was shown to be linked to the high-heparin regimen, whatever the nature of heparin (Figure 3). However, this study failed to demonstrate a significant correlation between elastase increase and heparin activity level (aXa). The more potent effect of the high-heparin dose over the low dose is concordant with the studies on PMN degranulation in vitro [14,15,17] and with recent reports showing a reduced PMN degranulation during dialysis with citrate as compared with UFH [9,10]. A potential advantage of LMWH over UFH known in vitro with superoxide ion production [15], cannot be confirmed in the present study in vivo. In addition, the effect on protein catabolism of this increase of elastase [34,35] may be more important with UFH than with LMWH. Indeed, UFH is known to be more strongly bound with elastase than LMWH, thus reducing the action of alpha-proteinase inhibitor [36]. We have measured that, at doses used usually in haemodialysis, both heparin types did not influence significantly the measurement of plasma elastase in complex with α1-proteinase inhibitor. Recently, however, it was shown in vitro that UFH, and LMWH to a lesser degree, directly inhibits the activity of elastase [37]. Unfortunately, the final effect of heparins on elastase is unknown in vivo because it is impossible to measure the specific biological activity of elastase, which is mixed with other serine proteinase activities. Finally, heparins could also act on amyloidogenesis, per se as a source of glycosaminoglycans, or as a factor of altered proteolysis by leukocyte stimulation [19,34,35].

The precise mechanism of heparin action on PMNs is not known. Our study allows the exclusion of an insufficient anticoagulation and subsequent clot formation mechanism, that can induce leukopenia but that cannot induce neutrophil degranulation, during in vitro haemodialysis [5]. Indeed, we observed the most stimulatory effect during the high-heparin regimen (Figures 2 and 3), and as a result a direct activating effect of heparins should be considered. At a concentration less than 2 IU/ml (aXa) observed during routine dialysis, UFH in vitro provokes an up-regulation of endothelial adhesion leukocyte receptors (beta 2-integrin) [12]. This receptor up-regulation is also induced by the dialysis membranes known to produce an in vivo leukopenia [38]. In addition, heparin can provoke a granulocyte degranulation in a calcium-dependent manner [15]. This heparin action on PMNs is thought to be due to a specific polysaccharide sequence, different from that of antithrombin III [16,39]. We propose that heparin could act in vivo on blood PMNs in synergy with the stimulatory effect of the dialysis membrane, by acting on membrane pre-activated cells. This membrane starter effect may be compared to that of i.v. infusion of phorbol myristate acetate, which facilitates the UFH-induced leukopenia in rabbits [17].

The PMN and elastase variation might also be affected by heparin upper levels and industrial production. The heparin concentration threshold (aXa) between the stimulatory action at low concentrations and the inhibitory effect at higher concentrations has rarely been studied. This threshold is estimated to be in vitro 1–2 IU/ml [11,12,15], mostly greater than regular heparin concentrations used in dialysis, and therefore in favour of the heparin stimulatory action during the dialysis session in the present study. Since we used the same batch for each type of heparin, a variable effect according to the batch of heparin [13,14] can also be ruled out.

The decrease in PMN count (Figure 2) and elastase concentration (Figure 3) that was observed in the second part of the dialysis session has also been
reported by other authors. Firstly, a comparable PMN variation was observed by Stegmayr et al. [40], using UFH and a modified cellulose membrane with a similar correction for haemocoagulation. In addition, the cellulose triacetate membrane we used has been shown to promote a more important granulocyte sequestration, as compared to cuprophane or polysulphone membranes [32]. Heparins are unlikely to be the cause of this variation, since it was shown in vitro that, at a concentration greater than 1 IU/mL UFH can provoke a down-regulation of endothelial leukocyte receptors (L-selectin) [12,16] though promoting the PMN increase after the initial granulocytopenia [38]. Secondly, the decrease in elastase concentration (Figure 3) was also observed by others with UFH and using a modified cellulose membrane [40] or a high-permeability polysulphone membrane [41]. These results are in agreement with the recent findings that the PMN degranulation can be mainly an early transient process, especially for primary granules [9] which contain elastase.

Finally, this study did not show any specific heparin action on lactoferrin (Figure 4), which confirms our previous results in vitro [15]. Hence, our study suggests that UFH and LMWH stimulate in vivo the degranulation of PMN primary granules containing elastase, but not the secondary granules containing lactoferrin.

**Conclusion**

In the present study, the heparin type and dose effects appear to be distinct from other potential extracorporeal circuit effects. A low-molecular-weight heparin (nadroparin calcium) used at regular high dose during a 4-h dialysis session induces a less granulocytopenic effect as compared with an usual high dose of unfractionated heparin. There was no protective effect of LMWH on neutrophil degranulation as measured by elastase release, but for both UFH and LMWH, an advantage of low dose over high dose is shown. Thus, from a biocompatibility point of view, it may be interesting to choose a low-molecular-weight heparin at a low dose. Furthermore, these specific effects of heparin nature and regimen should be considered in future trials addressing biocompatibility in maintenance haemodialysis.

**References**

17. Monreal M, Vinas L, Monreal L, Savin S, Lafos E, Angles AM. Low molecular weight heparin of IL8: C3 is not essential.

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