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Effects of heparin and derivatives on podocytes: An in vitro functional and morphological evaluation

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Abstract

Unfractionated heparin (UFH) and low molecular heparin derivatives (LMWH) display numerous biological properties in addition to their anticoagulant effects. However, due to the physicochemical heterogeneity of these drugs, a better understanding concerning their effects on human cells is clearly needed. Considering that heparins are mainly excreted by the kidney, we focused our attention on the effect of UFH and LMWH on human podocytes by functional and morphological/phenotypic in vitro analyses. We demonstrated that these products differentially modulate the permeability of podocyte monolayer to albumin. The functional perturbations observed were correlated to significant cellular morphological and cytoskeletal changes, as well as a decrease in the expression of proteins involved in podocyte adherence to the extracellular matrix or intercellular interactions. This point confirms that UFH and the different LMWHs exert specific effects on podocyte permeability and underlines the need of in vitro tests to evaluate new biological nonanticoagulant properties of LMWH.

KEYWORDS
heparin, low molecular heparin derivatives, podocyte, podocyte permeability

1 | INTRODUCTION

Unfractioned heparin (UFH) and its low molecular weight derivatives (LMWH) are widely used for the prevention and the treatment of venous thromboembolic events (Gray, Hogwood, & Mulloy, 2012). Heparins are heterogeneous glycosaminoglycans composed of a mixture of polysulfated chains comprised of alternating disaccharide residues of D-glucosamine and uronic acid residues linked by glycosidic bonds. Low molecular weight heparins (2–8 kDa) are issued from UFH (14 kDa) by chemical or enzymatic depolymerisation and possess different anticoagulant properties. In addition to their different polysaccharide size distribution, the structural variations could explain the different anticoagulant profiles of LMWH. These molecules show considerable variations in their pharmacokinetic and pharmacodynamic effect, arguing in favour of noninterchangeability of LMWH as recommended by the Food and Drug Administration

(Fareed et al., 2004). In addition to these anticoagulant properties, LMWHs display numerous other biological activities such as anti-inflammatory, antiangiogenic or antimetastatic properties (Yan et al., 2017).

UFH and derivatives are mainly excreted by the kidney. Numerous studies have been performed in chronic kidney disease (CKD) patients, allowing the establishment of specific posology and LMWH choice as a function of kidney filtration capacity (Sciascia et al., 2017). UFH is able to limit glomerular function deficiency in glomerulonephritis through multiple mechanisms involving an effect on growth factors, an inhibition of heparinase, or by attenuating the inflammatory status (Gambaro & Kong, 2010; Gambaro et al., 1992). Accordingly, sulodexide (a heterogeneous group of sulfated glycosaminoglycans) has demonstrated a beneficial impact on the proteinuria observed in diabetic patients; even its efficacy is still debated (Li et al., 2015; Olde Engberink, & Vogt, 2016). In contrast to the curative or

renoprotective effects of heparins, little is known about their effects on healthy kidney. In vitro studies have demonstrated that heparin could enhance specific gene expression in isolated rat podocytes (Yaoita et al., 2014); but up to now, there is no functional study analysing the effects of UFH or LMWH on the human kidney. The constant development of new pharmacological compounds, and in particular heparin derivatives, needs functional tests to be able to predict their effects on kidney function (Tiong et al., 2014).

Kidney glomerular filtration involves endothelial cells, glomerular basement membrane, and podocytes. Podocytes are terminally differentiated epithelial cells that display elongated interdigitating foot processes that form a zipper-like network allowing filtration. Specific interactions between cells, named slit diaphragms, form the ultimate filtration barrier which prevent the passage of macromolecules larger than serum albumin (Garg, 2018). Among the numerous proteins that are involved in the slit diaphragm, nephrin and ZO-1 play an important role in the maintenance of this particular structure. Besides these specialised intercellular complexes, podocytes must efficiently adhere to the basal membrane. At a molecular level, cell–matrix interactions involve podocyte adhesion receptors, such as integrins (and notably the main integrin $\alpha 3\beta 1$), that are intimately coupled to cell cytoskeleton (Sever & Schiffer, 2018). This podocyte spatial organisation is crucial for the maintenance of an efficient glomerular filtration. These properties identify the podocyte as a "weak link" in numerous pathologies such as diabetes mellitus (Dai, Liu, & Liu, 2017), lupus nephritis (Chen & Hu, 2017) or focal segmental glomerulosclerosis (FSG), as well as a sensitive target for potential nephrotoxic drugs.

Based on these considerations and previous results, we studied the effects of commercially available UFH and LMWH on podocytes. Considering that the podocyte is a sensitive target cell involved in glomerular filtration, we focused on this cell using an in vitro assay allowing the measurement of albumin permeability through a human podocyte monolayer. The goal of this study was to investigate the effect of heparin and its derivatives on podocyte permeability by functional tests combined with morphological/phenotypic analysis after drug exposure. Our in vitro approach has also been carried out to highlight new properties of heparins regardless of their anticoagulant capacities.

2 | MATERIALS AND METHODS

Except when specified, all the reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). UFH (Heparine sodique®; Panpharma SA, Luitré, France); the LMWH enoxaparin (Lovenox®; Sanofi Aventis, Lyon, France); tinzaparin (Innohep®; Leo Pharma, Voisins-le-bretonneux, France); and the direct oral anticoagulants dabigatran (Pradaxa®; Boehringer Ingelheim, Paris, France), rivaroxaban (Xarelto®; Bayer HealthCare SAS, Lyon, France) and argatroban (Arganova®; LFB Biomedicaments, Courtaboeuf, France) were all obtained in their commercial forms.

2.1 | Cell culture

Human podocytes, kindly provided by Saleem et al. (2002), were routinely cultured in RPMI-1640 medium containing 10% foetal bovine serum, 1% insulin–transferrin–selenium-A supplement, and 1% penicillin–streptomycin solution as previously described (Delézay et al., 2017). The cells were cultured at 33°C with 95% air and 5% CO_2 in 75 cm^2 flasks (BD Falcon; Le Pont de Claix, France). To obtain fully differentiated podocytes, the cells were switched to a 37°C incubator (5% CO_2) and cultured in the same medium for 12–14 d before use. For cultures on permeable supports, undifferentiated cells (grown at 33°C) were seeded at a density of 2.5×10^5 cell/filter (BD-Falcon cell culture inserts, 24-well size, 3-μm pore size), cultured 24 hr at 33°C for cell adhesion and then switched to 37°C for 12–14 d. The medium was changed twice a week.

2.2 | Evaluation of cell toxicity

Podocytes were seeded in 96-well plates at a density of 100,000 cells/well and allowed to differentiate at 37°C for 12 d. Then the cells were treated with different concentrations of each molecule during 2 d. Following incubation, cytotoxicity of the tested compounds was evaluated by both LDH and XTT assays. LDH release in cell supernatant was quantified using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI) and the XTT assay was performed using the In Vitro Toxicology Assay Kit, XTT based according to the manufacturer's instructions. Each condition was performed in quadruplicate ($n = 4$).

2.3 | Evaluation of podocyte monolayer permeability

The experiments were performed on differentiated podocytes grown on permeable supports (BD-Falcon cell culture inserts, 24-well size, 3-μm pore size) treated or not for 48 hr with increasing concentrations of UFH, enoxaparin, tinzaparin, fondaparinux, argatroban, rivaroxaban or dabigatran in the two compartments (apical and basal). Each assay was performed in quadruplicate ($n = 4$) with a positive control (puromycin aminonucleoside [PAN], 40 μg/ml) to verify podocyte response to drug injury. Podocyte monolayer permeability was determined in the basal to apical direction.

Cells were first incubated for 1 hr at 37°C in serum-free RPMI medium (SF-RPMI) and then incubated with fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) at a concentration of 0.5 g/L in the basal compartment (1 ml). After an incubation of 4 hr at 37°C, 100 μl was taken from the apical compartment, transferred into a 96-well plate and fluorescence was measured using a fluorimeter (Exc = 485 nm, Em = 538 nm; Fluoroskan Ascent; Thermo Fisher Scientific, Dardilly, France).

The apparent permeability of albumin was calculated with the following formula:

$$P_{app} = \frac{V_r}{C_0} \times \frac{1}{S} \times \frac{C_2}{t}$$

where P_{app} is the apparent permeability, V_r is the volume of medium solution in the receiving chamber, C_0 is the initial concentration of drug in the basal compartment, S is the area of the monolayer, C_2 is the concentration of the drug found in the apical compartment after an incubation of 4 hr, and t is the incubation time.

2.4 | Morphological/phenotypic analysis by immunofluorescence experiments

Immunofluorescence experiments were performed with phalloidin-iFluor 555 (Abcam, Cambridge, UK) and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Life Technologies, Carlsbad, CA) to visualise cell cytoskeleton and nucleus, respectively. Monoclonal antibodies directed against ZO-1 (BD Bioscience, Le Pont de Claix, France) or against the integrin α3β1 (Dako, les Ulis, France) were used to show intercellular contacts and cell-support interactions, respectively. Differentiated podocytes grown on glass coverslips were fixed with a 4% paraformaldehyde solution for 20 min, permeabilised with 0.2% Triton X-100 (20 min). After two washes with phosphate-buffered saline (PBS), the cells were incubated with primary antibodies (1/50) for 1 hr at room temperature, washed and incubated with the anti-mouse Alexa Fluor 488 conjugated secondary antibodies (1/200; Thermo Fisher Scientific, Illkirch, France). The images were acquired using an epifluorescence inverted microscope (IX81; Olympus, Tokyo, Japan) equipped with a cell imaging software (SoftImaging System GmbH, Munster, Germany).

2.5 | Cell surface measurements

Podocytes were seeded on a 24-well plate and cultured for 12 days at 37°C to obtain fully differentiated cells. The cells were treated or not with PAN (40 μg/ml) or UFH (200 UI/ml) for 48 hr. Live cells were rinsed with RPMI complete medium and then incubated with the same medium containing 2 μM of calcein acetoxymethyl (Calcein-AM; Interchim, Montluçon, France) and 8 μM of Hoechst 33342 (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 45 min at room temperature. After two rinses, the wells were observed with an epifluorescence inverted microscope (IX81; Olympus) allowing the observation of the entire well by using a FITC filter for observing calcein-AM labelling, or a DAPI filter for observing Hoechst 33342 labelling.

For the quantitative analysis of cellular parameters, 12 images (three images by well, four wells for each condition) were captured and analysed using the ImageJ software (Rueden et al. 2017) using a protocole adapted from the article by Kachurina et al. (2016). Briefly, the cell number was determined by counting Hoechst 33342-labelled cell nucleus (blue channel) using the "analyse particles" function on ImageJ software after an automatic threshold (default). For the quantification of the total cell surface, the scale was converted in μm (using the scale bar) and a bandpass filter was applied on each image (green channel) to homogenise the fluorescent labelling. The same automatic threshold (MinError)

was applied on each picture allowing the measurement of the total cell surface using the appropriate ImageJ software function (area measurement). The value obtained for each image was divided by the number of cells counted in the same field observed to obtain an average individual cell surface.

2.6 | Cell detachment assay

Differentiated podocytes grown on 96-well plate were incubated with or without PAN (40 μg/ml) or heparin (200 UI/ml) or enoxaparin (200 UI/ml) or tinzaparin (200 UI/ml) for 48 hr. Eight wells of untreated cells were rapidly fixed with 4% paraformaldehyde solution for 20 min (positive control of cell adhesion, 0% detachment) and eight wells were incubated 10 min with trypsin (negative control of cell adhesion, 100% detachment). Experimental wells were washed three times with PBS before to be fixed with the paraformaldehyde solution for 20 min. After three washings with water, the cells were stained with a 0.1% crystal violet in 2% ethanol for 60 min. The wells were then washed again with water in the dye was solubilised by using a 10% acetic acid solution. The absorbance was measured at 570 nm using a plate reader (Tecan, Lyon, France).

2.7 | Flow cytometry experiments

Cell cycle analyses were performed on undifferentiated or differentiated podocytes treated or not with UFH (200 UI/ml, 48 hr) or enoxaparin (200 UI/ml, 48 hr) or tinzaparin (200 UI/ml, 48 hr). After treatment, cell monolayers were disrupted by trypsin–ethylenediaminetetraacetic acid treatment. The cells were then incubated with a Hoescht solution (20 μM) for 30 min at 37°C, washed and analysed on a FACS Vantage cell sorter (BD Biosciences, San José, CA). Specific ratings were done to determine the proportion of proliferative and quiescent cells for each condition.

The detection of two parietal epithelial markers (Pax-2 and Claudin-1) was performed on differentiated cells after their treatments with UFH or enoxaparin or tinzaparin (200 UI/ml, 48 hr). The cells were dissociated with trypsin and then fixed and permeabilised by using paraformaldehyde (4% solution, 20 min) and Triton X-100 (0.2% solution, 20 min), respectively. After washings, cells were incubated with primary antibodies (1/100) directed against Pax-2 (anti-Pax-2 antibody; Abcam) or against Claudin-1 (Ozyme, Saint Quentin en Yvelines, France) during 1 hr. Detection of the first antibody was done by using anti-rabbit Alexa Fluor 488 conjugated secondary (1/200). The omission of the first antibody serves as a negative control (background signal) for the acquisition on FACS Vantage cell sorter.

2.8 | Evaluation of modulation of protein expression by cell-based immunoassay

Immunodetection of the proteins involved in cell–matrix interactions were performed with antibodies directed against CD29 (integrin β1;

Bio-Rad, Marnes-la-Coquette, France), CD49c (integrin α3; Bio-Rad), CD61 (integrin β3; Bio-Rad). Observation of expression of proteins involved in intercellular interactions was done by using antibodies directed against Nephrin (Sigma-Aldrich, Saint-Quentin Fallavier, France) or ZO-1 (BD Bioscience).

Differentiated podocytes grown in 96-well plates were treated or not with PAN (40 μg/ml), UFH (200 UI/ml), enoxaparin (200 UI/ml) or tinzaparin (200 UI/ml) for 48 hr before to be rapidly fixed and permeabilised with paraformaldheyde (4% in PBS) and Triton X-100 (0.2% in PBS), respectively. After incubation with RPMI complete medium to block unspecific binding sites, cells were incubated with the different antibodies (1/100 in RPMI complete medium) for 3 hr. After several washes, monoclonal antibodies binding was revealed with peroxidase-conjugated anti-mouse antibodies (1/500; CliniSciences, Nanterre, France) followed by 3,3′,5,5′-tetramethylbenzidine substrate. Optical density was quantified at 450 nm with a spectrophotometer (Tecan). The omission of primary antibodies served as control value that has been subtracted to the different experimental points values.

2.9 | Statistical analysis

All results are expressed as means ± standard deviations (SD). Results were compared using the nonparametric Mann–Whitney test. A $p < 0.05$ (*) was considered statistically significant.

3 | RESULTS

3.1 | Effect of heparin and LMWH on podocyte monolayer permeability

Initial experiments were done to analyse the effect of heparin, enoxaparin, tinzaparin and fondaparinux on podocyte monolayer permeability to fluorescent albumin. As shown in Figure 1a, heparin was able to enhance the apparent permeability of albumin. The effect was significant from a concentration of 50 UI/ml ($p < 0.05$) and the permeability increased with heparin concentration. The apparent permeability obtained with the highest concentration (400 UI/ml) was in the same order as the value obtained with the nephrotoxic drug PAN (Figure 1e). Similar data were obtained with enoxaparin
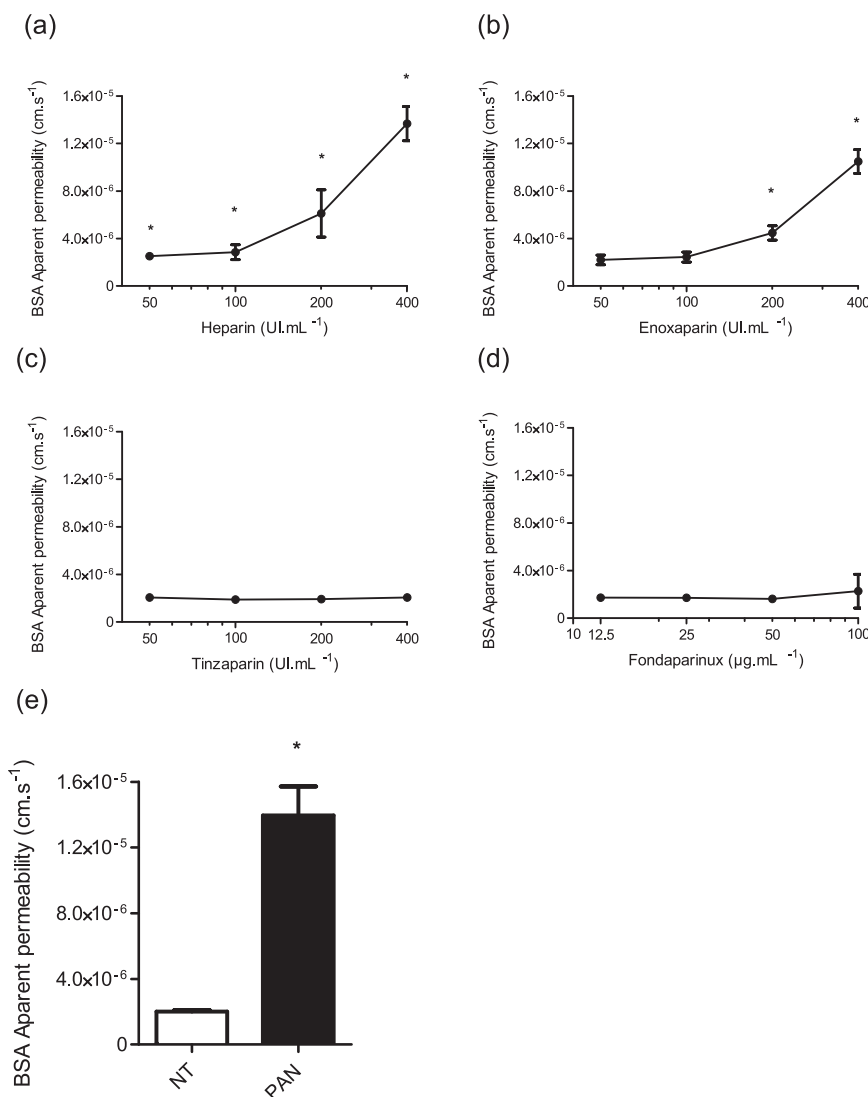
FIGURE 1 Effect of unfractionated heparin and low molecular heparin derivatives on podocyte permeability to albumin. Differentiated podocytes grown on permeable supports were incubated with increasing concentrations of unfractionated heparin (a), enoxaparin (b), tinzaparin (c) or fondaparinux (d) for 48 hr before being tested for albumin permeability. The nephrotoxic drug puromycin aminonucleoside (PAN, 40 μg/ml) was used as a positive control of podocyte perturbation (e). The results are expressed as a mean ± SD of four independent assays. Statistically significant differences between untreated cells and treated cells are calculated using the nonparametric Mann–Whitney test (*$p < 0.05$). BSA: bovine serum albumin

(Figure 1b), but the effect was significant only from a concentration of 200 UI/ml ($p < 0.05$). Similarly, an increase in enoxaparin concentration was associated with an increase of albumin permeability. In contrast to these results, tinzaparin (Figure 1c), fondaparinux (Figure 1d), used in similar conditions, were unable to modulate albumin permeability, suggesting relative specificity of this effect. One should note that whatever the concentration used, no cell toxicity could be observed using XTT and LDH assays (Supporting Information Figure S1).

3.2 | Effect of direct oral anticoagulants on podocyte permeability to albumin

Similar experiments were done with direct oral anticoagulants to check if the effect observed could be related to the anticoagulant properties of the compounds. After 48 hr of drug exposure, the permeability to albumin was evaluated. The results, presented in Figure 2, indicated that argatroban, dabigatran, or rivaroxaban were unable to increase albumin permeability despite the use of relatively high concentrations (Figure 2a–c). The use of PAN as a positive control for podocyte alteration indicated that the cells were nevertheless responsive. No cellular toxicity could be observed whatever the condition (Supporting Information Figure S1).

3.3 | Analysis of morphological changes

Immunofluorescent experiments were then performed to analyse potential cellular morphological changes after drug treatment. As shown in Figure 3, the treatment of cells with UFH (Figure 3b) was associated with a significant decrease in cell spreading (with the

presence of "hole areas") compared with the control cells (untreated; Figure 3a). The relatively homogeneous monolayer observed with untreated cells was clearly disorganised after UFH treatment, with cells displaying a compact morphology. This cellular shrinkage led to uncoupling from adjacent cells. The actin filament labelling was drastically modified and appeared concentrated around the nucleus, reflecting an important cell condensation. Similar results were obtained with enoxaparin treatment (Figure 3c), but this phenomenon was not observed for cells treated with tinzaparin (Figure 3d). Furthermore, treatments of the cells with fondaparinux or with argatroban did not show any podocyte morphological changes (data not shown), in agreement with functional permeability tests.

3.4 | Cell surface evaluation under treatments

The visualisation of these cell morphological changes prompted us to quantify the effects of UFH on cellular spreading and on cell detachment. Differentiated podocytes were treated for 48 hr with UFH (200 UI/ml) or with PAN (40 µg/ml) before to be incubated with calcein to obtain a homogeneous labelling of the live cells. As shown in Figure 4, the visualisation of the entire well demonstrated that the monolayer was affected after PAN treatment with the presence of a greater intercellular space compared with untreated monolayer. This macroscopic observation was also noticed to a lesser extend after heparin treatment. The observation at higher magnification clearly confirmed the presence of "holes" between cells after puromycin treatment. Cells treated with heparin also appeared slightly more distant from each other than untreated cells. However, in these conditions, the quantification of the number of cells, using ImageJ software on 12 different pictures for each condition, did not evidence
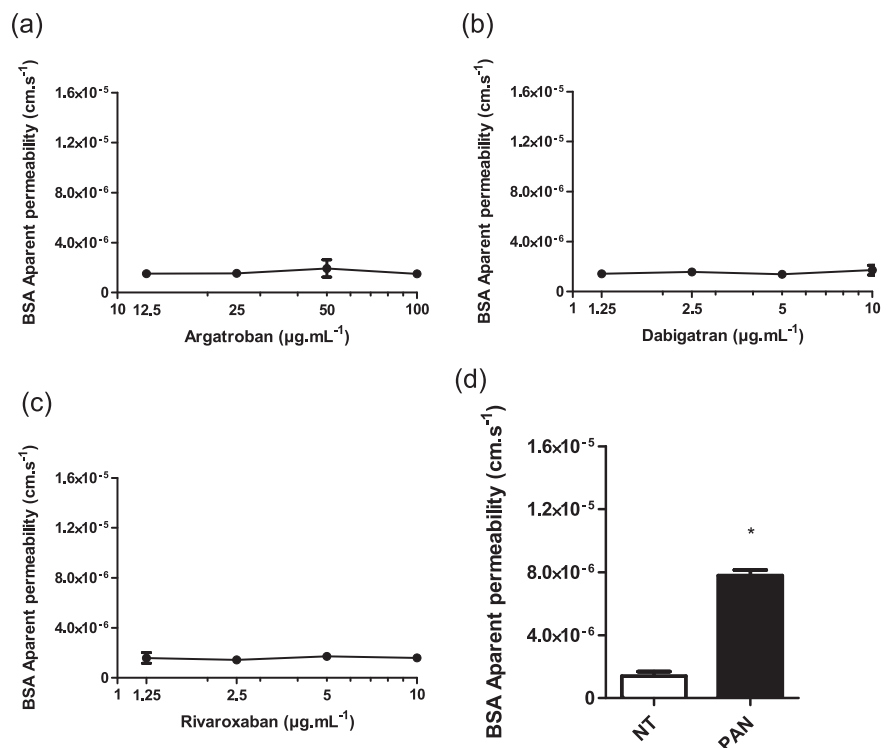
FIGURE 2 Effect of direct oral anticoagulants on podocyte permeability to albumin. Podocytes were incubated with different concentrations of dabigatran (a), argatroban (b), or rivaroxaban (c) for 48 hr before being tested for albumin permeability. Puromycin aminonucleoside (PAN) served as positive control of filtration alteration. The values are shown as the mean ± SD of four independent tests. Statistically significant differences between untreated cells and treated cells are calculated using the nonparametric Mann–Whitney test (*$p < 0.05$). BSA: bovine serum albumin

significant differences between untreated or UFH treated cells. In contrast, the surface of the cell monolayer and the surface of individual cell were significantly lower ($p < 0.005$) for UFH treated

cells compared with untreated cells. This result indicated that UFH treatment caused a "cell narrowing." Similar quantifications were performed on PAN treated cells. This drug, used as a positive control for monolayer disturbance, was associated, as expected, with a significant ($p < 0.0001$) decrease of the cell number compared with untreated cells confirming the ability of this drug to promote podocyte detachment. Accordingly, the total surface covered by the cells was diminished. However, the individual cell surface was also significantly decreased ($p < 0.0001$) indicating that the entire cell surface decrease was not only he fact of cell loss but also due to a narrowing of the individual cell surface.

In another experiment, the assessment of cellular detachment was performed on PAN, UFH, enoxaparin and tinzaparin treated cells (Supporting Information Figure S2). In contrast to the results mentioned above, heparin treatment was associated with a significant cell loss compared with untreated cells ($67.9 \pm 17\%$ vs. $36.5 \pm 15\%$ of detached cells for heparin and untreated cells, respectively). This result suggested that heparin weakened cell adhesion. Interestingly, enoxaparin did not affect significantly cell adhesion. The treatment with tinzaparin was quite different since this drug seemed to limit cell loss compared with untreated cells ($9 \pm 19\%$ vs $36.5 \pm 15\%$, respectively). As expected, PAN treatment generated a significant cell detachment inducing more than 79% of cell loss.

3.5 | Dedifferentiation evaluation

As previously described (Delézay et al., 2017), undifferentiated podocytes are smaller than differentiated ones that are spreading on the culture support. The morphological changes observed with UFH-treated differentiated cells prompted us to analyse the effect of this treatment on cellular dedifferentiation. Flow cytometry experiments were performed with undifferentiated or differentiated podocytes treated or not treated with UFH or enoxaparin or tinzaparin (200 UI/ml, 48 hr). As demonstrated in Figure 5, heparin treatment was not associated with a significant change in the cytograms obtained after Hoescht cell labelling. As expected, the proportion of proliferative cells was greater for undifferentiated podocytes than for differentiated cells (34.6% vs. 18.9% of the total cell population, respectively). After the different treatments, no significant increase of proliferating cells could be noticed on differentiated cells. The percentages were 22.4%, 21.8%, and 20.6% after heparin, enoxaparin, and tinzaparin treatments, respectively. These results suggested that UFH or LMWH treatments were not associated with an increase in proliferative capacity of the cells.
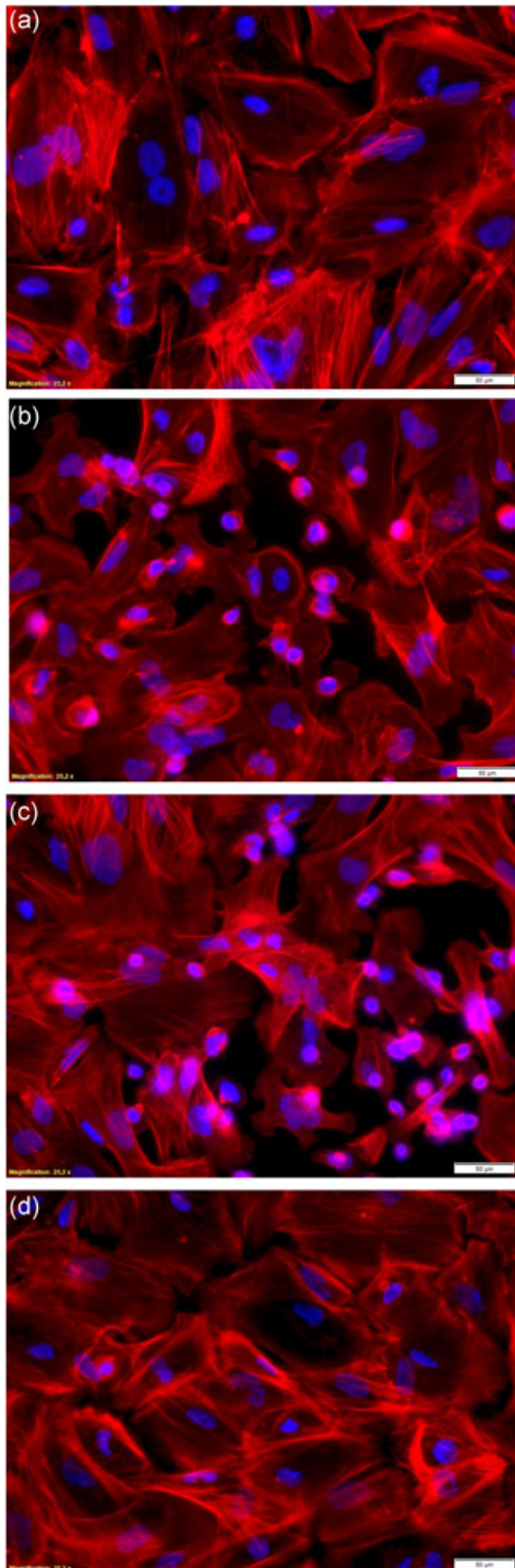
FIGURE 3 Morphological analysis of podocyte after treatment with UFH and low molecular heparin derivatives. Differentiated podocytes, grown on glass coverslips, were incubated or not (a) with 200 UI/ml of UFH (b), enoxaparin (c), or tinzaparin (d) for 48 hr before being fixed and permeabilised. Phalloidin-iFluor 555 was used to visualise the F-actin cytoskeleton component (in red) and DAPI was used for nucleus labelling (in blue). Scale bar = 50 μm. DAPI: 4′,6-diamidine-2′-phenylindole dihydrochloride; UFH: unfractionated heparin [Color figure can be viewed at wileyonlinelibrary.com]
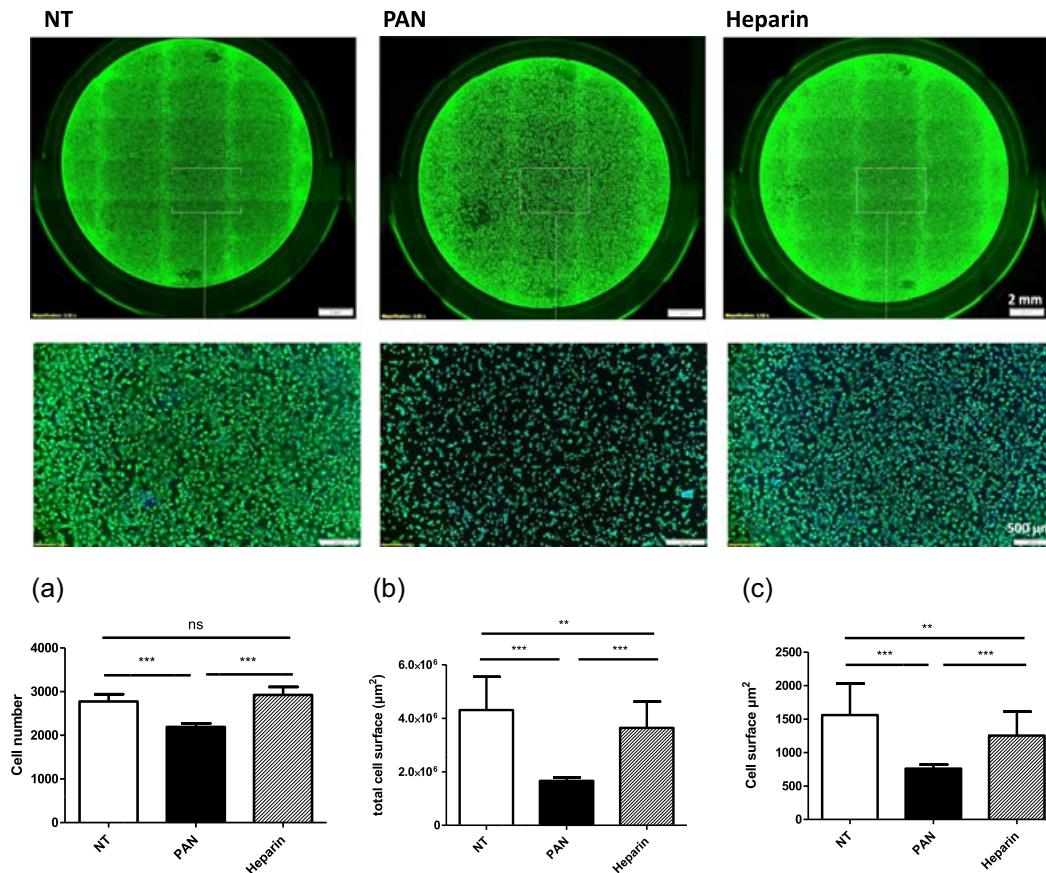
FIGURE 4 Quantitative analysis of cell surface after UFH treatment. Podocytes, cultured in 24-well plates, were treated with PAN (40 μg/ml) or UFH (200 UI/ml) for 48 hr before to be labelled using calcein. Live cells were visualised using an epifluorescence inverted microscope microscope (IX81; Olympus, Tokyo, Japan) allowing the observation of the entire well by using a FITC filter for observing calcein-AM labelling. For each condition, 12 pictures were randomly captured and analysed for cell number (a), total (b), or individual (c) cell surface. Statistically significant differences between the different conditions were estimated using the nonparametric Mann–Whitney test (***$p < 0.0005$; **$p < 0.005$). FITC: fluorescein isothiocyanate; *ns*: not significant; PAN: puromycin aminonucleoside; UFH: unfractionated heparin [Color figure can be viewed at wileyonlinelibrary.com]

To confirm these results, the expression of Claudin-1 and Pax-2, two markers of parietal epithelial cells, were analysed on differentiated podocytes after the different treatments. The results indicated that UFH, enoxaparin and tinzaparin (200 UI/ml, 48 hr) did not modulate the expression of these two markers (Supporting Information Figure S3) refuting the progression of these cells to a parietal cell phenotype.

3.6 | Effect of heparin on α3β1 integrin, ZO-1, and nephrin expression

Intercellular contacts between podocytes and cell matrix attachment are key elements for efficient glomerular filtration. ZO-1, nephrin, and the integrin α3β1 are, among a large panel of molecules, three proteins needed for cell–cell interactions and cell–matrix adhesion, respectively. Since UFH seems to modify the organisation of the podocyte monolayer, the expression of ZO-1 or α3β1 integrin or nephrin was investigated on untreated or UFH treated cells by immunofluorescent experiments. PAN treatment (40 μg/ml, 48 hr) was used as control for podocyte morphological perturbation. As

shown in Figure 6, PAN treatment was associated with a modification of α3β1 integrin distribution. The pattern was less homogeneous on cell surface and appeared diminished compared with untreated cells. Similar perturbations were evidenced for ZO-1 expression with the presence of rounded cells lacking ZO-1 expression on their cytoplasmic membrane. The detection of nephrin that display a punctiform fluorescent labelling on untreated cells was also affected with only few cells expressing this marker.

Heparin treatment (200 UI/ml, 48 hr) led also to a significant decrease in α3β1 integrin labelling intensity. Compared with untreated cells that displayed a homogeneous labelling on their plasma membranes, the expression of the integrin on treated cells was restricted to some parts of the plasma membrane or to the intracellular compartment. Similar observation was obtained with ZO-1 labelling. The typical plasma membrane detection of ZO-1 observed with untreated cells was totally abolished, with only few cells expressing ZO-1 expression on their plasma membrane. Nephrin was detected on heparin treated cells but the signal was clearly lower and more heterogeneous than for untreated cells.
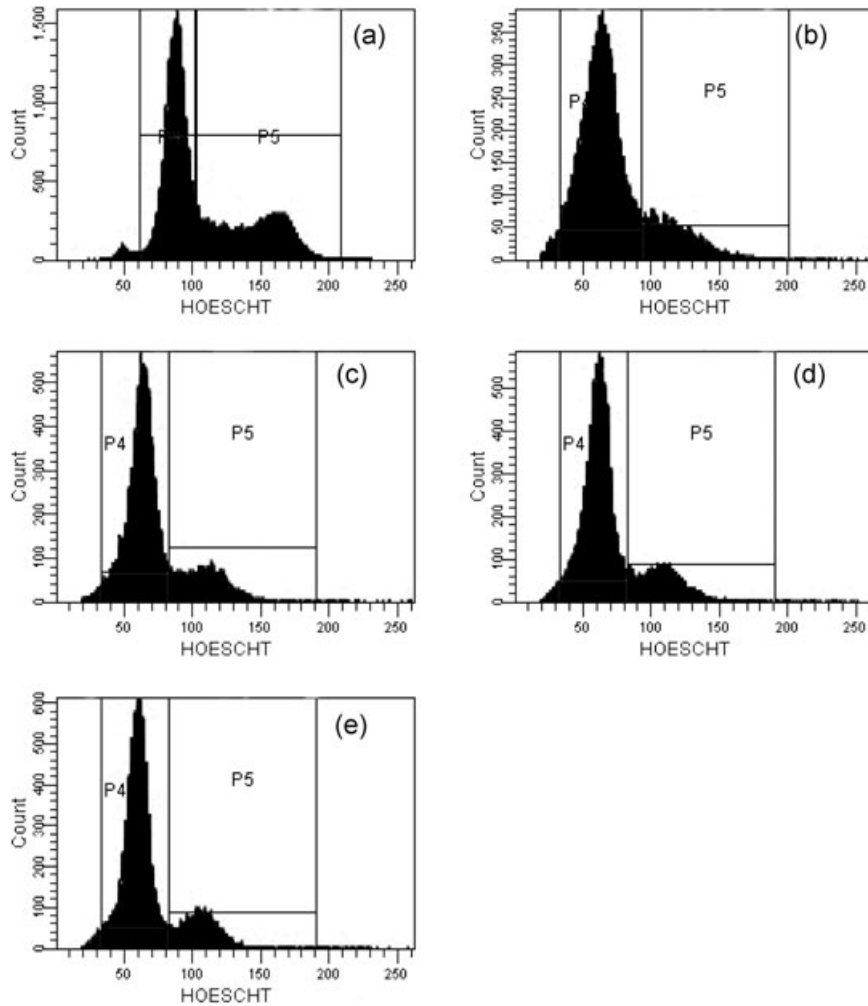
FIGURE 5 Flow cytometry study of podocyte replication properties after unfractionated heparin (UFH) or low molecular heparin derivative (LMWH) treatments. Undifferentiated (a) and differentiated (b–e) podocytes were grown in 25 cm^2 flasks and treated or not with 200 UI/ml of UFH (c) or enoxaparin (d) or tinzaparin (e) for 48 hr before monolayer disruption and cell incubation with Hoescht solution (20 μM, 30 min, 37°C). Cell cycle analysis was performed on a fluorescence-activated cell sorting (FACS) Vantage cell sorter (BD Biosciences, San José, CA). P4 and P5 gating allowed the quantification of quiescent or replicative cells, respectively

3.7 | Quantification of integrins, nephrin and ZO-1 expression by cell-based immunoassays

To quantify the variation observed in immunofluorescence experiments, a semiquantitative cell-based immunoassay was performed on untreated cells or cells treated with UFH (200 UI/ml), enoxaparin (200 UI/ml) or tinzaparin (200 UI/ml) for 48 hr. As illustrated in Figure 7, all the proteins tested were significantly decreased by UFH treatment compared with untreated cells. Similar effects were observed with enoxaparin, except for CD61 (integrin β3) expression which was not significantly modified. The most pronounced effect of UFH was observed for the expressions of nephrin and ZO-1, that were diminished by 41% and 45%, respectively, compared with control cells. The expression of the β1 (CD29) and α3 (CD49c) integrins were also affected and decreased by 22% and 32%, respectively, compared with the untreated cells. The effect of enoxaparin displayed a similar profile with a significant decrease in nephrin and ZO-1 expressions (−44% and −48% in comparison to untreated cells, respectively). Interestingly, the effect of tinzaparin was very different with ZO-1 expression slightly diminished (−17% compared with untreated cells). The expressions of the markers CD29 and nephrin were not modified. In contrast, α3 (CD49c) and

β3 (CD61) were increased compared with untreated cells (+7% and +33%, respectively). These results confirmed that enoxaparin and tinzaparin act differently on podocytes. PAN treatment, used as control, significantly diminished the expression of the different markers tested (Supporting Information Figure S4).

4 | DISCUSSION

UFH and LMWH have long been used for the prevention or treatment of thrombotic events and are more recently studied for their nonanticoagulant properties such as anti-inflammatory, anti-angiogenic, or antimetastatic effects (Yan et al., 2017). These new properties offer interesting perspectives but need the evaluation of these compounds on specific target cells to explore their potential beneficial or deleterious effects. The fact that these drugs are mainly excreted by the kidney prompted us to explore their effects on podocyte, a major actor of the glomerular filtration.

We analysed the effect of UFH or LMWH on podocyte monolayer permeability by using an in vitro human podocyte model allowing a functional test combined with morphological/phenotypic analysis.
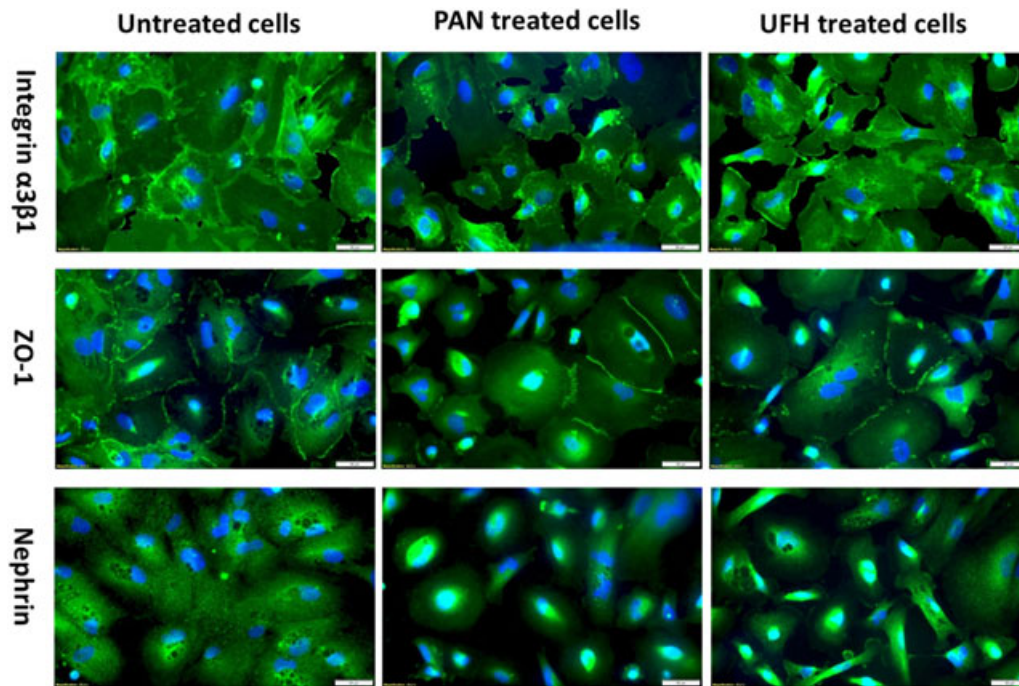
FIGURE 6 Modulation of the expression of nephrin, α3β1 integrin, and ZO-1 by PAN or UFH treatment. Differentiated podocytes, grown on glass coverslips, were treated or not with UFH (200 UI/ml, 48 hr) or with PAN (40 μg/ml) before being fixed and permeabilised. Cells were incubated with antibodies directed against nephrin, α3β1 integrin, or ZO-1, which were revealed (in green) by using Alexa Fluor 488 conjugated goat anti-mouse IgG antibodies. Nucleus staining was performed with DAPI (in blue). Omission of the primary antibody served as negative control. Scale bar = 20 μm. DAPI: 4′,6-diamidino-2-phenylindole; IgG: immunoglobulin G; PAN: puromycin aminonucleoside; UFH: unfractionated heparin [Color figure can be viewed at wileyonlinelibrary.com]

Our data demonstrated that UFH and enoxaparin were able to increase the permeability of podocytes to albumin in contrast to tinzaparin or fondaparinux or direct oral anticoagulants. The effect was not associated with cell toxicity as evidenced by LDH or XTT assays, despite a high range of concentrations (in agreement with the results obtained by Yaoita et al.). Immunofluorescent studies revealed that the deleterious effect on podocyte permeability was correlated with morphological perturbations and phenotypic changes. Quantification of cell number and cell surface parameters indicated that UFH caused a cellular shrinking. UFH weakened cell adhesion as evidenced in detachment assay experiment. Finally, cell-based immunoassay experiments demonstrated that UFH and enoxaparin only were able to decrease the expression of proteins involved in podocyte adhesion or in cell–cell interactions.

In accordance with previous results (Delézay et al., 2017), this study confirmed that UFH increases the permeability of albumin through a podocyte monolayer. This effect correlated with the concentration of UFH with an apparent permeability of albumin reaching the values obtained with the nephrotoxic drug PAN. Similar data were obtained with enoxaparin. Interestingly, tinzaparin or fondaparinux had no effect on podocyte permeability. This first result indicated that enoxaparin and tinzaparin, both derived from UFH, do not possess the same biological activity on these glomerular epithelial cells. This result also suggested that this effect was not directly associated with the anticoagulant property of the molecule. This point is furthermore reinforced by the use of another class of molecules (direct oral anticoagulant compounds) that are unable to modulate albumin permeability in our in vitro model. The discrepancy observed between enoxaparin and tinzaparin effects on podocyte filtration function may be due to different manufacturing processes. Tinzaparin is the only heparin derivative that is obtained by an enzymatic digestion (heparanase); while enoxaparin is obtained after benzylation and alkaline depolymerisation of heparin. Therefore, the two drugs possess different physicochemical properties, such as a different molecular weight distribution (Bisio et al., 2015) or sulfation. The proportion of low molecular weight polysaccharides (<2,000 Da) is greater for enoxaparin (12–20%) than for tinzaparin (<10%). These considerations could explain the differences observed in our functional test and could be compared with those obtained by Bârzu et al. (1986) on endothelial cells. In the same way, tinzaparin and enoxaparin displayed different antiangiogenic properties (Mousa, 2013) or different antifibrotic effects in the liver (Abdel-Salam, Baiuomy, Ameen, & Hassan, 2005) for example. The fact that the effect observed on podocyte permeability is only obtained at high concentrations, far from the concentrations used in clinical use, may suggests that the "active molecule(s)" is (are) probably present in small amounts in the heterogeneous mixtures of heparin and enoxaparin.

The particular organisation of podocyte monolayer is a key element for an efficient glomerular filtration. Then, we have analysed morphological and phenotypic changes that could be induced by UFH or LMWH treatments.
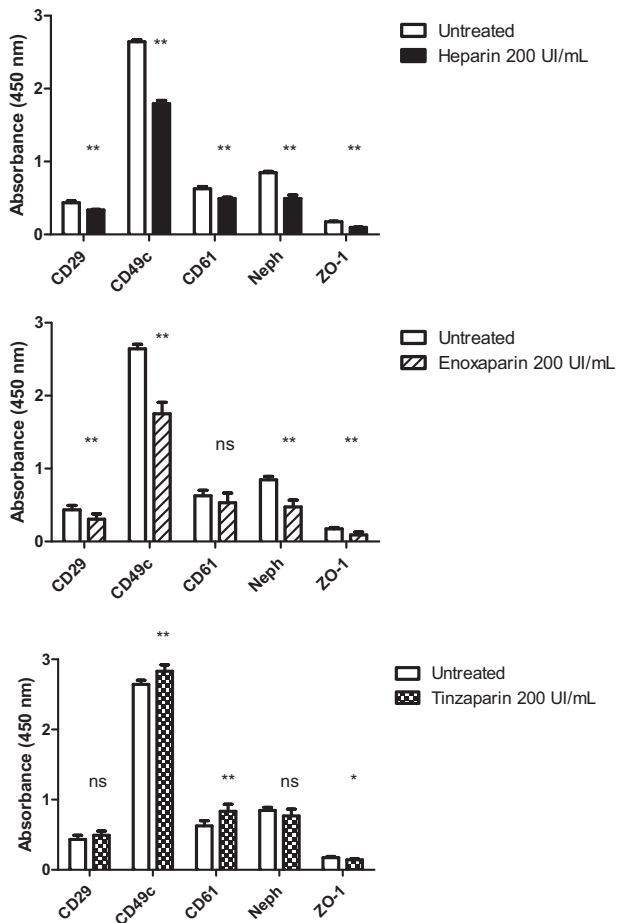
FIGURE 7 Effect of (UFH or low molecular heparin derivatives on protein expression revealed by a cell-based immunoassay. Differentiated podocytes, cultured in 96-well plates, were treated or not with 200 UI/ml of UFH or enoxaparin or tinzaparin for 2 d. After the fixation and permeabilisation steps, cells were incubated with the specified antibodies that were revealed with HRP-conjugated anti-mouse antibodies and 3,3′,5,5′-tetramethylbenzidine as substrate. Optical density (absorbance) measurements were performed with a spectrophotometer (450 nm). Values reported are the mean ± SD of six independent assays. *$p < 0.05$, **$p < 0.01$, ($p > 0.05$). HRP: horseradish peroxidase; *ns*: not significant; UFH: unfractionated heparin

Immunofluorescent experiments demonstrated that enoxaparin and UFH (but not tinzaparin, fondaprinux or argatroban) treatments were able to modify cellular morphology. In particular, the actin fibres (visualised by using fluorescent phalloidin) appeared clearly condensed compared with untreated cells, indicating that the treatment of the podocytes with the two drugs provoked an alteration of the cell cytoskeleton. The podocyte cytoskeleton is a key for efficient glomerular filtration (Welsh & Saleem, 2011) and this result can explain the increase in permeability to albumin observed in our functional assays after UFH and enoxaparin treatments. Other immunofluorescent experiments demonstrated a decrease in expression of α3β1 integrin, nephrin and ZO-1 proteins after UFH treatment, proteins that are critical for the adhesion of podocytes to the glomerular basement membrane (Chen et al., 2006; Pozzi et al., 2008) or intercellular

interactions. These contacts, between the elongated interdigitating foot processes, are assured by specific proteins such as nephrin, ZO-1 and podocin, which are closely associated with the cell cytoskeleton (Conti, Perico, Grahammer, & Huber, 2017). The decrease in expression of α3β1 integrin combined with the downregulation of ZO-1 and nephrin may explain the alteration in cellular morphology observed after UFH treatment, leading to larger monolayer permeability. The quantification of cell surface revealed that UFH treatment generated a significant cellular shrinking. This effect is comparable to that obtained after puromycin aminonucleoside treatment of the cells, although the latter is much more important. The analysis of cell adhesion confirms that UFH treatment weakens cell attachment. This cell condensation, enhancing the intercellular spaces, associated with cell loss is probably the main cause of podocyte permeability increase after heparin treatment.

Confirming immunofluorescent experiments, we found that the treatment of podocytes with UFH or enoxaparin resulted in a significant quantitative decrease in ZO-1, α3 and β1 markers and was also responsible for a decrease in β3 and nephrin proteins. Tinzaparin did not significantly modify the expression of nephrin and induced only a moderate decrease in ZO-1 expression. In addition, conversely to the effect obtained with UFH or enoxaparin, tinzaparin increased the expression of α3 and β3 markers. This last point can explain the fact that tinzaparin significantly enhances cell adhesion as evidenced in cellular detachment assays results. In addition, these data highlight different or even opposite effects of these products, which may explain the differences observed in the functional tests.

Taken together, our data indicate that UFH and enoxaparin perturb podocyte filtration property, probably by modifying the cytoskeleton and/or decreasing the expression of specific proteins involved in podocyte attachment and cell–cell interactions inducing a the decrease of the cellular spreading. This is not the case with tinzaparin. However, it is quite difficult to distinguish the chronological and the relative part of these two events in the perturbation of the podocyte permeability. Considering that glomerular filtration does not involve only podocytes but is also dependent on endothelial cells as well as a glomerular basement membrane, the evaluation of the effects of UFH and LMWH on a more complex in vitro system including these two parameters will be of particular relevance.

The intracellular mechanisms leading to podocyte injury by UFH or enoxaparin remain to be elucidated. A potential lead is the intracellular calcium path, since the podocyte cytoskeleton is highly sensitive to intracellular calcium modulations (Greka & Mundel, 2012). This hypothesis is supported by the fact that heparin can inhibit IP3-induced Ca^{2+} release (Nilsson, Zwiller, Boynton, & Berggren, 1988) and interfere with SDF-1 binding to CXCR4 (Ma et al., 2012), limiting the increase of intracellular concentration of calcium induced by SDF-1.

Finally, our study demonstrated that UFH and enoxaparin can deregulate podocyte permeability. Furthermore, our data indicate that the different LMWHs possess specific biological nonanticoagulant effects, depending on the manufacturing process involved in the elaboration of the drug. This new property, unrelated to the anticoagulant effect of these products, confirms that in vitro tests represent interesting tools for the evaluation of new biological

properties of LMWH. Our in vitro system, allowing functional and morphological approaches, could be used for the screening of several drugs that can interact with the podocyte.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

O. D., X. D., and Z. H. designed the experiments. O. D., Z. H., O. S., V. B., and S. H. performed the experiments. O. D., X. D., and P. M. wrote the paper. O. D., Z. H., O. S., S. H., V. B., M. S., P. M., and X. D. revised the manuscript.

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