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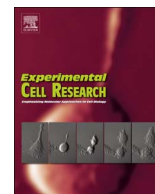
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## Glomerular filtration drug injury: In vitro evaluation of functional and morphological podocyte perturbations

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### ABSTRACT

The kidney is an organ that plays a major role in the excretion of numerous compounds such as drugs and chemicals. However, a great number of pharmacological molecules are nephrotoxic, affecting the efficiency of the treatment and increasing morbidity or mortality. Focusing on glomerular filtration, we propose in this study a simple and reproducible in vitro human model that is able to bring to light a functional podocyte injury, correlated with morphologic/phenotypic changes after drug exposure. This model was used for the evaluation of paracellular permeability of FITC-dextran molecules as well as FITC-BSA after different treatments. Puromycin aminonucleoside and adriamycin, compounds known to induce proteinuria in vivo and that serve here as positive nephrotoxic drug controls, were able to induce an important increase in fluorescent probe passage through the cell monolayer. Different molecules were then evaluated for their potential effect on podocyte filtration. Our results demonstrated that a drug effect could be time dependent, stable or scalable and relatively specific. Immunofluorescence studies indicated that these functional perturbations were due to cytoskeletal perturbations, monolayer disassembly or could be correlated with a decrease in nephrin expression and/or ZO-1 re-location. Taken together, our results demonstrated that this in vitro human model represents an interesting tool for the screening of the renal toxicity of drugs.

### 1. Introduction

The kidney represents an important target for drug-induced toxicity since it is responsible for the excretion of numerous xenobiotics and is continuously exposed to drugs. The filtering unit of the kidney, the nephron, contains capillaries whose walls function as a filtration barrier. In addition to the glomerular endothelial cells, the filtration process implies specialised cells, the podocytes that cover the capillary. The structural organisation of the podocyte that displays an interdigitating structure (foot process) assures a selective filtration of molecules depending on their size [1]. Considering that the cut-off is around 70 kDa, the finding of albumin in urine is the first symptom of glomerular filtration dysfunction that could be observed in numerous pathologies [2]. Disorganisation of the podocyte architecture using nephrotoxic drugs, such as puromycin aminonucleoside (PAN) or adriamycin (ADR), results in an increase of protein barrier crossing,

confirming the involvement of this cell as a major actor of glomerular filtration [3,4]. There is a need of simple relevant models to evaluate the effects of pharmacological drugs on glomerular filtration, to screen new compounds and to elucidate cellular events involved in functional perturbations. Numerous rodent models [5,6] have been developed to study kidney physiology and functionality, but recently research is focusing on the podocyte considering that this cell is the “weak link” in the filtration process [7]. In vitro approaches have been studied using rodent podocytes, but the establishment of a human differentiation inducible cell line by M. Saleem offers new perspectives for the study of the human podocyte [8]. This cell line has been already used for fundamental research [9] as well as for the study of cellular events involved in kidney pathologies [10].

Considering that such a model could be used for the screening of numerous compounds, we chose a simple, standardisable approach focusing on the podocyte to evaluate the effects of pharmacological

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compounds on glomerular filtration. The goal of our study was to set up an *in vitro* human podocyte model in order to be able to predict the deleterious effect of drugs on glomerular filtration. Selective glomerular filtration was determined by measuring the apparent permeability of 70 kDa dextran molecules or BSA, before and after drug exposure. The analysis of morphological modifications was done to correlate functional perturbations to phenotypic/morphological changes.

## 2. Materials and methods

### 2.1. Cell culture

Except when specified, all the reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Podocytes were routinely cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS), 1% insulin-transferrin-selenium-A supplement, and 1% penicillin/streptomycin solution. The confluent monolayer was regularly disrupted by using a trypsin-EDTA solution.

The immortalised human podocytes were routinely cultured at 33 °C with 95% air and 5% CO<sub>2</sub> in 75 cm<sup>2</sup> flasks (BD Falcon, Le Pont de Claix, France). To obtain fully differentiated podocytes, the cells were switched to a 37 °C incubator (5% CO<sub>2</sub>) and cultured in the same medium for 12–14 d.

For cultures on permeable supports, undifferentiated cells (grown at 33 °C) were seeded at a density of 2.5 × 10<sup>5</sup> cell per filter (BD-Falcon cell culture inserts, 24-well size, 3 μm pore size), cultured 24 h at 33 °C for cell adhesion and then switched to 37 °C for 12–14 d. Medium was changed twice a week.

Caco-2 cells, approved by the FDA for *in vitro* transport studies, were chosen as a positive control for epithelium tightness since these cells display a significant transepithelial electrical resistance (TEER) when cultured on permeable support. These cells, grown on DMEM high glucose medium supplemented with FBS (10%) and 1% penicillin/streptomycin solution, were seeded at a density of 10<sup>5</sup> cells per filter (3 μm pore size BD Falcon inserts, 24-well plate format). These cells were used when the TEER (measured with an EVOM resistance meter; World Precision Instrument, Sarasota, USA) reached a value in the range of 400 Ω cm<sup>2</sup>.

### 2.2. Apparent permeability measurements

The evaluation of selective filtration was done with four FITC-dextrans possessing different molecular weights (4, 20, 70 and 150 kDa). Apparent permeability 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 drug found in the apical compartment after an incubation of 4 h, and  $t$  is the incubation time.

The experiments were performed with undifferentiated cells, differentiated podocytes or Caco-2 cells (control of epithelial tightness). Podocyte filtration functionality was determined in the basal to apical direction. Cells were first incubated for 1 h at 37 °C in serum-free RPMI medium (SF-RPMI) and then incubated with FITC-dextran or FITC-BSA at a concentration of 0.5 g L<sup>-1</sup> in the basal compartment (1 mL). After an incubation of 4 h at 37 °C, 100 μL were 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, ThermoFisher Scientific, Dardilly, France). All the assays were performed in quadruplicates (n = 4).

### 2.3. Modulation of podocyte transport by drugs

Drug effect on podocyte permeability was evaluated by using FITC-BSA, the gold standard protein for determination of glomerular filtration efficiency. The two known nephrotoxic drugs PAN and adriamycin ADR were used as positive controls for podocyte injury. Different compounds including risedronate (Rise), gentamycin (Gent), vancomycin (Vanco), ketoprofen (Keto), heparin were evaluated for their capacity to modulate podocyte filtration function.

Podocyte filtration was determined in the basal to apical direction with the same procedure as described above. The epithelial crossing of FITC-BSA was then performed as described above.

In addition, a high and a low concentration were tested during several days (3, 7 or 10 d). The apparent permeability of the podocytes was evaluated at the end of the incubation time. The two concentrations were chosen in agreement with the literature [11–13] and after preliminary assays were done to evaluate the highest nontoxic dose for each compound in our podocyte model. Untreated and PAN-treated cells served as negative and positive control, respectively, for podocyte dysfunction. Each experiment was done in quadruplicate (n = 4).

### 2.4. Cytotoxicity studies

Cell toxicity was evaluated by using the lactate dehydrogenase (LDH) release test that was purchased from Promega Corporation (Madison, USA). The experiments were performed according to the manufacturer's instructions.

### 2.5. Immunofluorescence studies

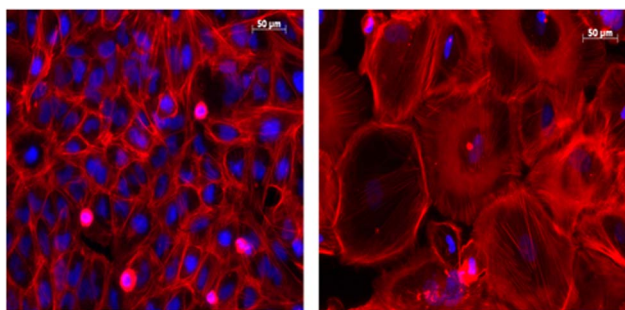
Immunofluorescence experiments were performed with Phalloidin-iFluor 555 (Abcam, Cambridge, UK) and 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; Life Technologies, Carlsbad, USA) to visualise cell cytoskeleton and nucleus, respectively. Mouse anti-ZO-1 antibody (clone 1) and mouse anti-nephrin antibody (clone 174CT2.1.1), used to visualise cell-cell contact complexes, were purchased from BD Bioscience (Le Pont de Claix, France) and from Sigma-Aldrich, respectively. Anti-mouse Alexa Fluor 488 conjugated secondary antibodies, used to detect primary antibodies, were obtained from Life Technologies.

Differentiated podocytes grown on glass coverslips were fixed with paraformaldehyde (3.7% in PBS) and permeabilised with Triton X-100 (0.5%). Then the cells were incubated with primary antibodies directed against nephrin (1/50) or ZO-1 (1/50) for 1 h at room temperature, washed and incubated with the anti-mouse Alexa Fluor 488 conjugated secondary antibodies (1/200) in the presence of Phalloidin-iFluor 555 and DAPI to visualise F-actin and cell nucleus, respectively. The images were acquired using an epifluorescence inverted microscope (IX81, Olympus, Tokyo, Japan) equipped with a cell imaging software (Soft Imaging System GmbH, Munster, Germany).

## 3. Results

### 3.1. Setup of the podocyte *in vitro* model: morphological aspects

Fig. 1 illustrates the cell line cultured at 33 °C (undifferentiated cell, left panel) or at 37 °C (differentiated cells, right panel). Undifferentiated cells formed a monolayer of relatively small cells with an intracytoplasmic peripheral pattern of actin staining. In contrast, differentiated podocytes appeared as large spreading cells that possess an important and multidirectional actin staining with small cytoplasmic extensions between cells resembling the specific foot process structure that is observed *in vivo*.



**Fig. 1.** Morphological aspect of undifferentiated (left panel) and differentiated podocytes (right panel). Cells grown on glass coverslips were fixed, permeabilised and incubated with Phalloidin-iFluor 555 and DAPI before being observed with a fluorescent microscope. In addition to an important difference in cell size, F-actin staining (in red) revealed different patterns of localisation.

### 3.2. Evaluation of filtration properties of the podocyte model

The functionality of our podocyte cells was evaluated by measuring the apparent permeability parameter with a range of molecular weight dextran molecules and BSA, as illustrated in Fig. 2.

Fig. 2A shows that passage of dextran through podocyte cell monolayer was dependent on the size of the molecules since the apparent permeability of the 4 kDa dextran was 5.3-fold and 25.2-fold higher than for the 20 kDa and 70 kDa dextrans, respectively. The  $P_{app}$  for 70 kDa was 4.8-fold higher with undifferentiated cells than the value obtained with differentiated cells. Cell differentiation was associated with a decrease in apparent permeability. As expected, Caco-2 cells, the positive control for epithelial tightness, displayed a reduced  $P_{app}$ .

Fig. 2B confirms that BSA apparent permeability was also dependent on podocyte cell differentiation. The apparent permeability of BSA with undifferentiated cells was 10-fold higher than the apparent permeability obtained for differentiated cells ( $3.7 \cdot 10^{-6} \pm 1.1 \cdot 10^{-6} \text{ cm s}^{-1}$  vs.  $2.3 \cdot 10^{-7} \pm 1.1 \cdot 10^{-8} \text{ cm s}^{-1}$ , respectively). Interestingly, the apparent permeability of BSA obtained with Caco-2 cells, ( $3 \cdot 10^{-6} \pm 5.9 \cdot 10^{-7} \text{ cm s}^{-1}$ ) was higher than the value obtained for differentiated podocytes.

### 3.3. Impact of nephrotoxic drugs on selective filtration

As expected, the treatment of podocytes for 24 h with PAN increased the epithelial crossing of the FITC-labelled molecule by 3.8 times and by 10-fold for 10 and 50  $\text{mg L}^{-1}$ , respectively. The same results were obtained with ADR with an increase in  $P_{app}$  of 13-fold with

the highest ADR concentration ( $150 \mu\text{g L}^{-1}$ ). The study of the effect of these nephrotoxic drugs by using FITC-BSA instead of FITC-dextran displayed similar results (Fig. 3B).

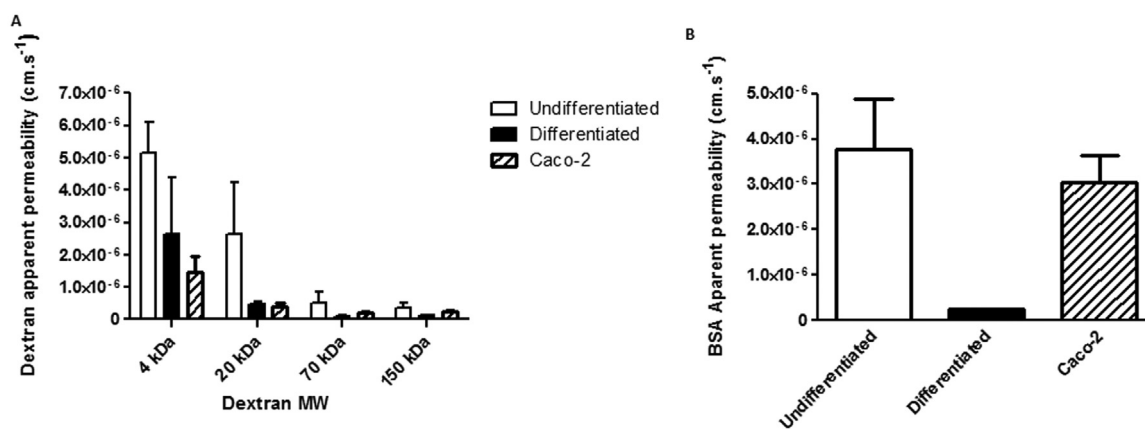
### 3.4. Evaluation of pharmacological drugs on podocyte selective filtration

Rise (a bisphosphonate), Gent and Vanco (two antibiotics), Keto (a nonsteroidal anti-inflammatory molecule) and heparin were evaluated in our model to predict their potential podocyte injury. The concentrations were chosen in agreement with the literature and with preliminary assays which were done. One exposure for 24 h did not show any significant change on BSA apparent permeability (Supplemental Fig. S1), suggesting that the potential deleterious effect of these drugs on podocyte filtration needs a longer treatment time. Podocyte filtration was then evaluated after 3, 7 or 10 days with a high (Fig. 4A) or a low (Fig. 4B) dose of the considered compound.

The highest concentrations of Gent and Rise were able to increase the apparent permeability of BSA (Fig. 4A), in contrast to the results obtained with low doses that were unable to modulate the permeability of BSA across the podocyte barrier (Fig. 4B). In the case of Gent, the effect was time dependent, reaching a value of 49-fold the value obtained with control cells after 3 exposures (10 d of treatment). The effect of Rise on podocyte filtration was earlier and relatively stable during time. In contrast to these drugs, Vanco or Keto did not alter podocyte filtration, suggesting a relative specificity of the effects obtained with Gent and Rise on the epithelial crossing of albumin. Heparin ( $200 \cdot 10^3 \text{ U L}^{-1}$ ) was able to significantly enhance BSA permeability after 3 d. This effect increased during time (Fig. 4C). LDH release measurements indicated that none of the treatments were toxic since any difference in LDH release could be noticed between treated or untreated cells in the supernatants (data not shown).

### 3.5. Evaluation of morphological changes induced by drugs

The expression and distribution of F-actin, ZO-1 and nephrin were analysed on differentiated podocytes treated for 3 d with the different drugs. The effect of the two nephrotoxic drugs (PAN and ADR) are shown in Fig. 5. The treatment of the cells with PAN was associated with a redistribution of the F-actin network that was located near the cytoplasmic membrane instead of the homogeneous distribution observed for untreated cells. ADR treatment was also associated with such a phenomenon as well as in cell-cell contact disruption with the appearance of isolated rounded cells. ADR treatment induced a decrease in cell size, with the cells resembling undifferentiated rounded cells. The labelling of ZO-1 on untreated cells was continuous along cell borders with some interdigitating signals at the cell-cell contacts. This



**Fig. 2.** Evaluation of FITC-dextran or FITC-BSA apparent permeability on undifferentiated and differentiated podocytes or on Caco-2 cells. Cells grown on permeable supports were tested for their permeability properties by using FITC-dextran possessing different molecular weights (A) or FITC-BSA (B) on undifferentiated cells (white bars) differentiated podocytes (blackbars) or Caco-2 cells (hatched bars). Results are expressed as the mean  $\pm$  SD of the apparent permeability ( $\text{cm s}^{-1}$ ) of four independent experiments.

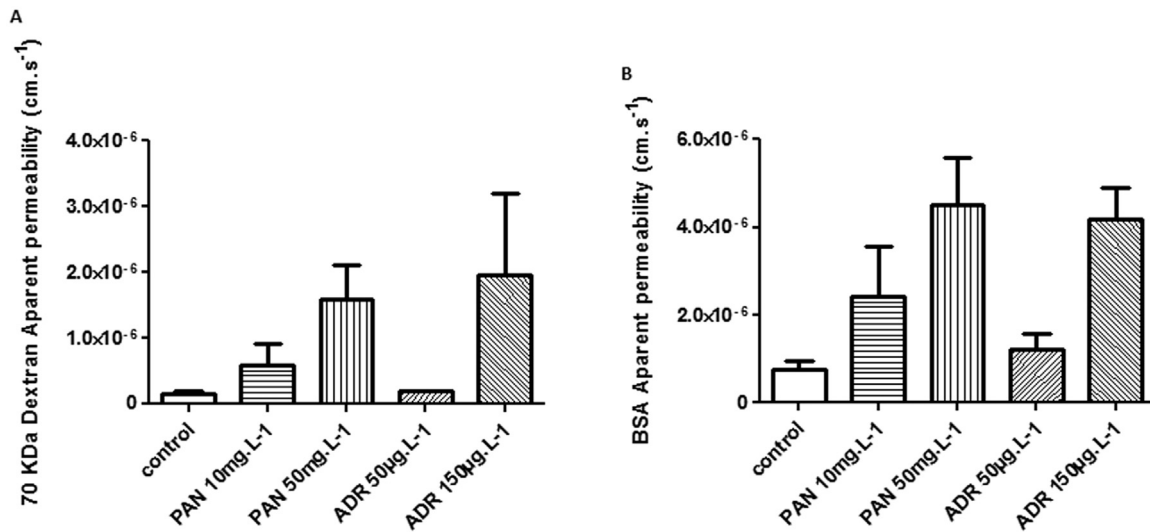


Fig. 3. Evaluation of permeability changes induced by drugs. Differentiated podocytes grown on permeable support were incubated for 24 h with puromycin aminonucleoside (PAN) or adriamycin (ADR) for the indicated concentrations before being evaluated for dextran (A) or BSA (B) permeability. The values are shown as the mean  $\pm$  SD of four independent assays.

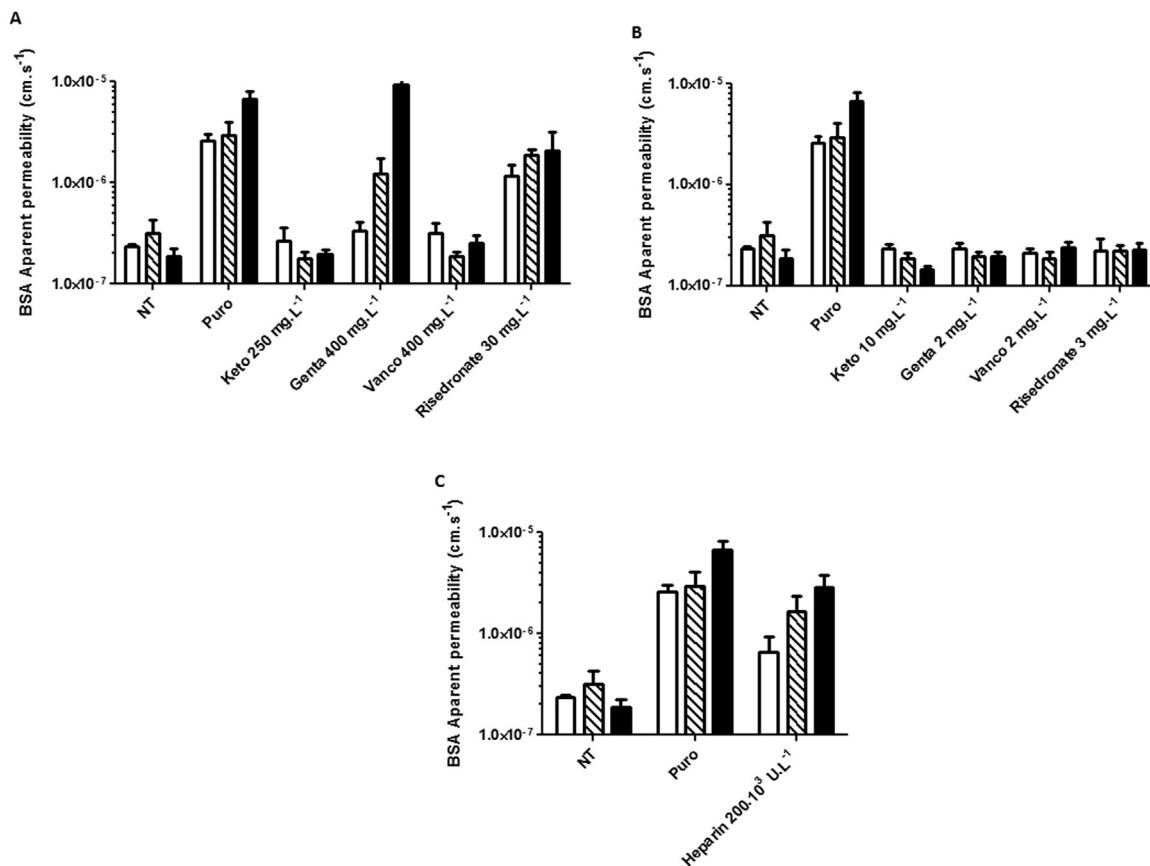


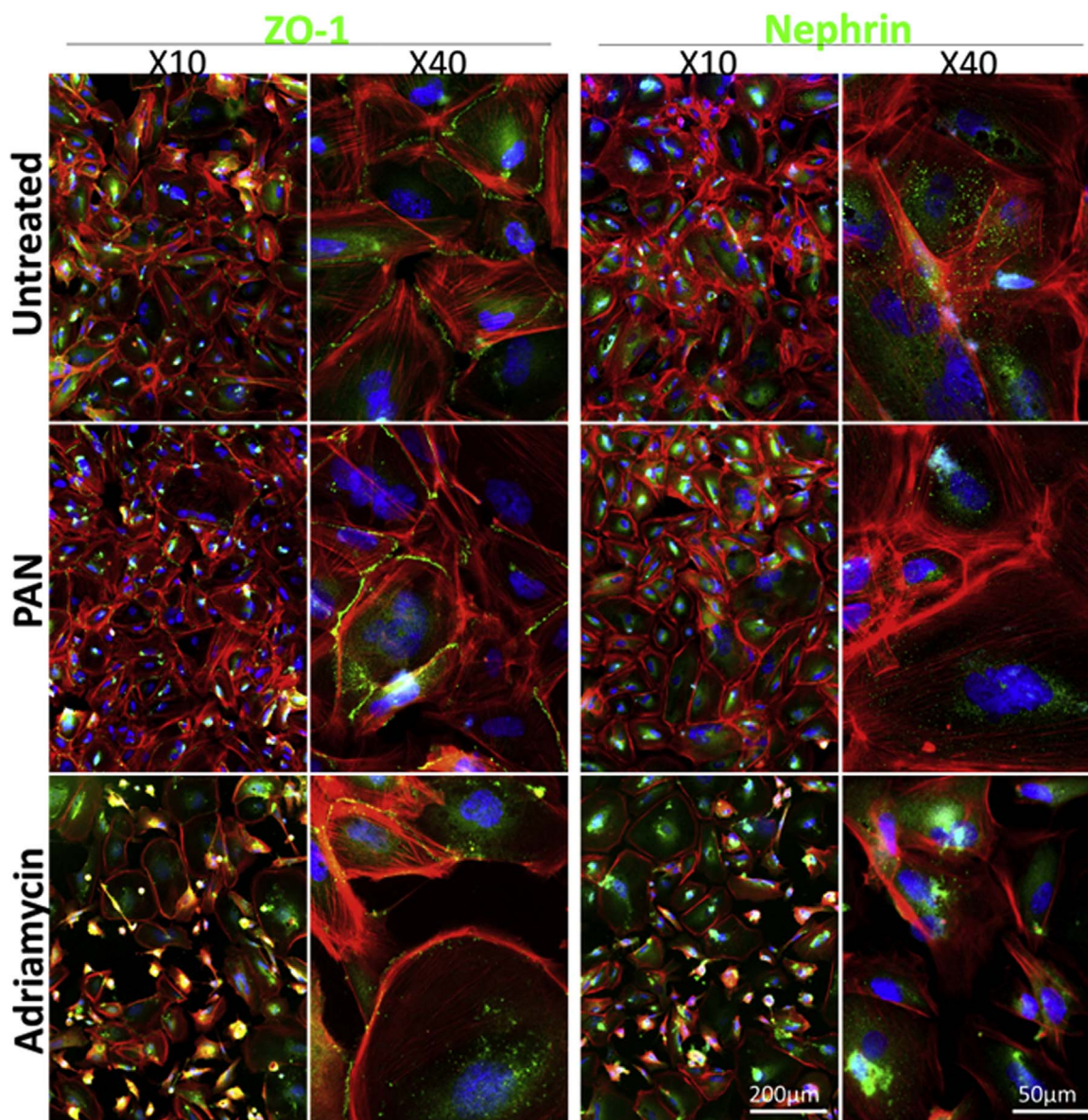
Fig. 4. Evaluation of drug effect on BSA permeability. Podocytes were grown on permeable support and then incubated with the high (A) or low (B) concentrations of different drugs for 3 (white bars), 7 (hatched bars) or 10 (black bars) days before being evaluated for BSA permeability. Untreated cells served as negative control and puromycin aminonucleoside was used as a positive control for monolayer filtration perturbation. The results are expressed as the mean  $\pm$  SD of four independent experiments.

pattern was identical on PAN-treated cells in contrast to the signal obtained after ADR treatment that resulted in a decrease in interdigitating structures associated with a redistribution of the protein on cell plasma membrane as patches. The expression of the nephrin protein was strongly decreased, resulting in a loss of the dot signal or in the dot and intracellular signals for PAN or ADR treated cells, respectively.

Similar immunofluorescence studies (Fig. 6) were conducted on podocytes treated for 7 d with Gent (400 mg L<sup>-1</sup>), Rise (30 mg L<sup>-1</sup>),

and heparin (200.10<sup>3</sup> U L<sup>-1</sup>). Gent treatment was associated with an important relocation of actin that was found near the cytoplasmic membrane and resembled the pattern observed with PAN-treated cells. Moreover, important spaces between cells appeared, suggesting cell-cell contact disruption. However, the expression of ZO-1 appeared to be unaffected by Gent in contrast to nephrin expression that was clearly reduced. The bisphosphonate Rise also provoked monolayer dissociation with the appearance of small rounded isolated cells. The loss of





**Fig. 5.** Effects of puromycin aminonucleoside (PAN) and adriamycin (ADR) on podocyte cytoskeleton, ZO-1 and nephrin expressions. Differentiated cells grown on glass coverslips were treated or not (NT) with PAN ( $10 \text{ mg L}^{-1}$ ) or ADR ( $100 \mu\text{g L}^{-1}$ ) for 3 d before being fixed and permeabilised. Phalloidin-iFluor 555 was used to visualise the F-actin cytoskeleton component (in red). DAPI was used for nucleus labelling (in blue). Cells were also incubated with antibodies directed against ZO-1 or nephrin that were revealed (in green) by using AF488 conjugated goat anti-mouse IgG antibodies. Omission of the primary antibody served as negative control.

cell-cell contact was also observed with the ZO-1 labelling, which was visualised as patches between separating cells instead of linear or interdigitating patterns observed between cells in close contact. This treatment was also associated with a dramatic decrease in nephrin expression, since very few cells displayed the specific dot pattern labelling. Similarly, heparin was able to dissociate the cells in some areas with the appearance of rounded cells. However, in residual confluent cell areas that were similar to those observed for untreated cells, the ZO-1 pattern appeared unchanged as compared to control cells. This was also the case for the expression of nephrin that was unchanged in confluent cell areas.

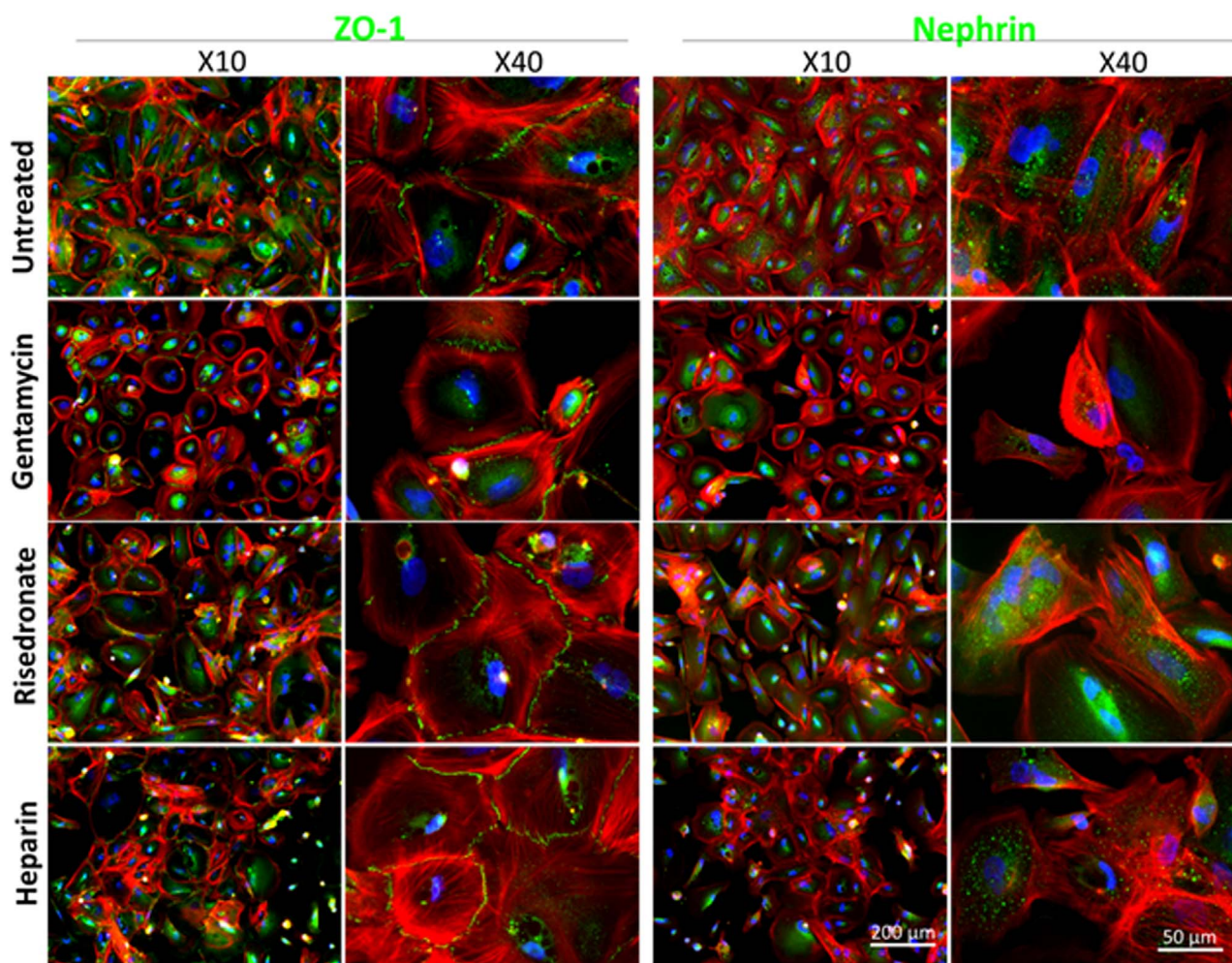
#### 4. Discussion

Drug-induced nephrotoxicity is a major problem in clinical human therapy since the use of nephrotoxic drugs is often unavoidable. Thus, the prediction of drug nephrotoxicity appears of particular relevance in order to avoid kidney injury and/or perturbation of pharmacokinetic

drug properties that are closely related to kidney excretion [14]. Moreover, the possibility to optimise drug design by using a functional screening in vitro model represents an interesting tool for the characterization of drug toxicity and/or for the development of new drugs. Numerous in vivo or in vitro models have been developed to explore the glomerular filtration [15]. However, these models are not always representative of human physiology (interspecies variations of in vivo models) or could not be easily used for the screening of drugs because of their complexity or costs (ex vivo approach).

In this study, we have used a simple standardised human in vitro model in order to measure the effect of different drugs on podocyte glomerular filtration. The setup of the model was based on the culture of human podocytes [8] on permeable support allowing the measurement of dextran or BSA epithelial crossing. Immunofluorescence experiments were done to analyse the morphologic/phenotypic changes induced by different treatments. To our knowledge, this is the first time that such approach is done for the evaluation of the nephrotoxicity of drugs with a focus on the glomerular filtration. In the great majority of





**Fig. 6.** Visualisation of phenotypic and morphologic changes induced by drug treatments. Podocytes were cultured during 7 d with gentamycin ( $400 \text{ mg L}^{-1}$ ), risedronate ( $30 \text{ mg L}^{-1}$ ) or heparin ( $200.10^3 \text{ U L}^{-1}$ ) before being fixed and permeabilised. Cells were then incubated with DAPI (blue) and phalloidin (red) to visualise cell nucleus or F-actin cytoskeleton components, respectively. Cells were also incubated with antibodies directed against ZO-1 or nephrin that were revealed (in green) with AF488 conjugated goat anti-mouse IgG antibodies. Omission of the primary antibody served as negative control.

cases, *in vitro* nephrotoxicity assays are performed by using proximal tubular cells without considering the podocyte that represents, nevertheless, a potential target for drugs. Our model appears to be closed to the functional state of normal human renal cells displaying a selective filtration that could be modulated by nephrotoxic drugs (PAN and ADR) known to induce proteinuria *in vivo* [16,17]. In addition, the analysis of F-actin distribution and nephrin or ZO-1 expression indicates a good correlation between functional and morphologic events due to treatments with these two drugs. In the absence of electron microscopic experiments, we cannot formally demonstrate that differentiated podocytes form slit diaphragms in our hands but the fact that podocyte monolayer displays a selective filtration and that this process could be perturbed by PAN and ADR, known to provoke foot process effacement, argue in favour of this point. The use of the phenotypic analysis combined with a functional test that reveals drug injury represents an innovative tool for the evaluation of drug injury.

Based on these considerations, we used this approach to test a panel of different molecules. We selected two antibiotics (Gent and Vanco), a bisphosphonate (Rise) a nonsteroidal anti-inflammatory drug (Keto) and heparin. These compounds were chosen since they could exert a nephrotoxicity but little is known about their actions on the glomerular filtration.

Gent, an aminoglycoside, could act on the proximal tubular part of the kidney, inducing an apoptosis [18] or a necrosis [19] of the epithelial cells. In the glomeruli, Gent could also promote mesangial

contraction [20], resulting in a decrease in glomerular filtration. Vanco, that belongs to the class of the glycopeptides, could provoke interstitial nephritis [21]. In the two cases, the effect of these molecules on podocytes is not documented. Non-steroidal anti-inflammatory drugs could also exert a nephrotoxicity modulating the vasoconstriction of afferent blood vessels [22,23]. Heparin has been described as a compound able to modulate the expression of specific proteins expressed by podocyte suggesting a potential effect on the glomerular filtration [24]. Bisphosphonates, used to reduce bone resorption in osteoporosis or in the cases of osteolytic bone metastases, are known to provoke tubular necrosis and collapsing focal segmental glomerulosclerosis [25]. They could act directly on podocytes, leading to a dedifferentiation and a proliferation of the cells [26]. Since the nephrotoxic effects could be dose and duration dependent, podocytes were continuously exposed to a low or a high concentration and tested for BSA transport after 3,7 or 10 days.

Our results clearly demonstrated that treatment of podocytes with Rise, Gent and heparin was able to modulate the permeability of BSA across the podocyte monolayer. The perturbation of podocyte filtration by drugs was only observed with the high concentrations, indicating that the filtration property was relatively resistant to long time exposure with low drug concentrations. The monolayer disruption, the perturbation of actin cytoskeleton and/or the decrease in nephrin expression could explain, at least in part, this result. It should be noted that whatever the treatment, no cellular toxicity could be observed by

LDH release measurements. An interesting finding was that these effects were time dependent, since some drugs were able to alter quickly the podocyte function (Rise, heparin), while others exerted a deleterious effect after a longer time exposure (Gent). Moreover, the functional injury appeared relatively stable during time for Rise or, in contrast, was scalable for Gent and heparin. Gent is known as a nephrotoxic drug that acts on proximal tubular cells. Interestingly, our data demonstrated that Gent could also alter glomerular filtration through a direct action on the podocyte. In our experiments, the effect of Gent was greater than that obtained with PAN after 10 d of treatment, suggesting an important deleterious action of this antibiotic on glomerular filtration. This unexpected data suggest that our functional test could highlight new mechanisms that could explain the nephrotoxicity of drugs. This effect appeared to be relatively specific since Vanco, used in the same conditions, was unable to promote a significant increase in BSA permeability. This result corroborates previous data that indicated that Vanco could be responsible for interstitial nephritis [21] rather than a direct action on podocytes. Similarly, Keto does not modify the filtration, in agreement with the fact that nonsteroidal anti-inflammatory compounds affect glomerular filtration rate by acting on blood vessels. These results indicate that our model is selective and, obviously, could differentiate drugs according to their cellular targets. The treatment of podocytes with the bisphosphonate Rise resulted in a rapid and significant increase in albumin epithelial crossing. Our immunofluorescence experiments showed monolayer disruption, the appearance of rounded isolated cells resembling undifferentiated podocytes, as well as a decrease in nephrin expression, which are in agreement with an effect of Rise on cell differentiation status. Heparin was also able to increase the epithelial crossing of BSA, suggesting that it could alter podocyte filtration function. This result is not in agreement with the results obtained by Yaoita et al. that demonstrated an increase in nephrin expression after heparin cell treatment, suggesting a potential benefit of heparin on glomerular filtration [24]. Despite these potential discordances, our results indicated that heparin is responsible for monolayer disruption rather than nephrin or ZO-1 down modulation, since confluent cell monolayer was comparable to that observed for untreated cells. This point indicates that functional tests are an important complementarity approach to protein expression studies and are pertinent to evaluating the drug effect. Other studies have reported a reduction of proteinuria after heparin treatment in PAN or ADR injured rats [27,28]. These results demonstrated that heparin could restore glomerular filtration function after drug injury. Since heparin could act at numerous levels in the kidney filtration process, it will be interesting to explore its effects in our cellular in vitro model to better understand its action on the podocyte. In particular, it will be interesting to evaluate the effect of heparin on podocyte adhesion by the measurement of integrin expression. Experiments will be performed in our laboratory to elucidate this point.

We think that our approach, using a simplified in vitro model, is suitable for the rapid screening of numerous pharmacological compounds as well as for the evaluation of the potential nephrotoxicity of new drugs. This point is reinforced by the fact that immunofluorescence experiments, that display monolayer organisation changes, variations in cytoskeleton distribution or protein expressions, are correlated with functional data. To our knowledge, this is the first time that the combination of functional and morphological studies is done to evaluate the nephrotoxicity of pharmacological compounds. However, the glomerular filtration is not only the fact of podocyte but is also the result of the fenestrated endothelial cells and the basal membrane. It will be pertinent to improve this model with endothelial cells. Nevertheless, since glomerular filtration deficiency often correlates with podocyte injury, we focused our study on this cell. Our simplified podocyte model could be of real interest for the prediction of drug-induced “podocytopathy” before clinical tests as well as for the optimisation of drug design. It also could be used for the screening of compounds that could restore podocyte filtration properties after podocyte injury.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2017.10.031>.

## References

- [1] F. Gramhammer, New structural insights into podocyte biology, *Cell Tissue Res.* 369 (2017) 5–10, <http://dx.doi.org/10.1007/s00441-017-2590-3>.
- [2] F. Cellesi, M. Li, M.P. Rastaldi, Podocyte injury and repair mechanisms, *Curr. Opin. Nephrol. Hypertens.* 24 (2015) 239–244, <http://dx.doi.org/10.1097/MNH.0000000000000124>.
- [3] T.-S. Ha, H.-Y. Park, S.-B. Seong, H.Y. Ahn, Puromycin aminonucleoside increases podocyte permeability by modulating ZO-1 in an oxidative stress-dependent manner, *Exp. Cell Res.* 340 (2016) 139–149, <http://dx.doi.org/10.1016/j.yexcr.2015.12.001>.
- [4] M. Yi, L. Zhang, Y. Liu, M.J. Livingston, J.-K. Chen, N.S. Nahman, F. Liu, Z. Dong, Autophagy is activated to protect against podocyte injury in adriamycin-induced nephropathy, *Am. J. Physiol. Ren. Physiol.* (2017), <http://dx.doi.org/10.1152/ajprenal.00114.2017>.
- [5] H.Y. Tiong, P. Huang, S. Xiong, Y. Li, A. Vathsala, D. Zink, Drug-induced nephrotoxicity: clinical impact and preclinical in vitro models, *Mol. Pharm.* 11 (2014) 1933–1948, <http://dx.doi.org/10.1021/mp400720w>.
- [6] G. Gambaro, A.O. Cavazzana, P. Luzzi, A. Piccoli, A. Borsatti, G. Crepaldi, E. Marchi, A.P. Venturini, B. Baggio, Glycosaminoglycans prevent morphological renal alterations and albuminuria in diabetic rats, *Kidney Int.* 42 (1992) 285–291.
- [7] J.S. Lin, K. Susztak, Podocytes: the weakest link in diabetic kidney disease? *Curr. Diab. Rep.* 16 (2016) 45, <http://dx.doi.org/10.1007/s11892-016-0735-5>.
- [8] M.A. Saleem, M.J. O'Hare, J. Reiser, R.J. Coward, C.D. Inward, T. Farren, C.Y. Xing, L. Ni, P.W. Mathieson, P. Mundel, A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression, *J. Am. Soc. Nephrol.* 13 (2002) 630–638.
- [9] C. Rigotherier, M.A. Saleem, C. Bourget, P.W. Mathieson, C. Combe, G.I. Welsh, Nuclear translocation of IQGAP1 protein upon exposure to puromycin aminonucleoside in cultured human podocytes: ERK pathway involvement, *Cell. Signal.* 28 (2016) 1470–1478, <http://dx.doi.org/10.1016/j.cellsig.2016.06.017>.
- [10] N. Chandel, B. Sharma, M. Husain, D. Salhan, T. Singh, P. Rai, P.W. Mathieson, M.A. Saleem, A. Malhotra, P.C. Singhal, HIV compromises integrity of the podocyte actin cytoskeleton through downregulation of the vitamin D receptor, *Am. J. Physiol. Ren. Physiol.* 304 (2013) F1347–F1357, <http://dx.doi.org/10.1152/ajprenal.00717.2012>.
- [11] S.C.T. Silva, L.A. de Almeida, S. Soares, M.F. Grossi, A.M.S. Valente, C.A. Tagliati, In vitro study of putative genomic biomarkers of nephrotoxicity through differential gene expression using gentamicin, *Toxicol. Mech. Methods* 27 (2017) 435–441, <http://dx.doi.org/10.1080/15376516.2017.1313345>.
- [12] Y. Sakamoto, T. Yano, Y. Hanada, A. Takeshita, F. Inagaki, S. Masuda, N. Matsunaga, S. Koyanagi, S. Ohdo, Vancomycin induces reactive oxygen species-dependent apoptosis via mitochondrial cardiolipin peroxidation in renal tubular epithelial cells, *Eur. J. Pharmacol.* 800 (2017) 48–56, <http://dx.doi.org/10.1016/j.ejphar.2017.02.025>.
- [13] E. Yaoita, Y. Yoshida, M. Nameta, Y. Zhang, H. Fujinaka, S. Magdeldin, B. Xu, T. Yamamoto, Heparin increasing podocyte-specific gene expressions, *Nephrology* 19 (2014) 195–201, <http://dx.doi.org/10.1111/nep.12207>.
- [14] B. George, D. You, M.S. Joy, L.M. Aleksunes, Xenobiotic transporters and kidney injury, *Adv. Drug Deliv. Rev.* (2017), <http://dx.doi.org/10.1016/j.addr.2017.01.005>.
- [15] D. Scotcher, C. Jones, M. Posada, A. Rostami-Hodjegan, A. Galetin, Key to opening kidney for in vitro-in vivo extrapolation entrance in health and disease: part I: in vitro systems and physiological data, *AAPS J.* 18 (2016) 1067–1081, <http://dx.doi.org/10.1208/s12248-016-9942-x>.
- [16] N. Dong, L. Meng, R. Xue, M. Yu, Z. Zhao, X. Liu, Adrenomedullin ameliorates podocyte injury induced by puromycin aminonucleoside in vitro and in vivo through modulation of Rho GTPases, *Int. Urol. Nephrol.* (2017), <http://dx.doi.org/10.1007/s11255-017-1622-y>.
- [17] N. Wang, R. Wei, P. Li, Q. Li, X. Yang, Y. Yang, M. Huang, R. Wang, Z. Yin, Y. Lv, X. Chen, Treatment with irbesatan may improve slit diaphragm alterations in rats with adriamycin-induced nephropathy, *J. Renin-Angiotensin-Aldosterone Syst. JRAAS* 17 (2016), <http://dx.doi.org/10.1177/1470320316646884>.
- [18] P. Randjelovic, S. Veljkovic, N. Stojiljkovic, D. Sokolovic, I. Ilic, Gentamicin nephrotoxicity in animals: current knowledge and future perspectives, *EXCLI J.* 16 (2017) 388–399, <http://dx.doi.org/10.17179/excli2017-165>.
- [19] X.H. Du, C.L. Yang, Mechanism of gentamicin nephrotoxicity in rats and the protective effect of zinc-induced metallothionein synthesis, *Nephrol. Dial. Transplant.* 9 (Suppl 4) (1994) 135–140.
- [20] C. Martínez-Salgado, F.J. López-Hernández, J.M. López-Novoa, Glomerular nephrotoxicity of aminoglycosides, *Toxicol. Appl. Pharmacol.* 223 (2007) 86–98, <http://dx.doi.org/10.1016/j.taap.2007.05.004>.
- [21] O. Bamgbola, Review of vancomycin-induced renal toxicity: an update, *Ther. Adv. Endocrinol. Metab.* 7 (2016) 136–147, <http://dx.doi.org/10.1177/2042018816638223>.
- [22] M.A. Perazella, Drug-induced nephropathy: an update, *Expert Opin. Drug Saf.* 4 (2005) 689–706, <http://dx.doi.org/10.1517/14740338.4.4.689>.
- [23] G.S. Pazhayattil, A.C. Shirali, Drug-induced impairment of renal function, *Int. J. Nephrol. Renov. Dis.* 7 (2014) 457–468, <http://dx.doi.org/10.2147/IJNRD.S39747>.



- [24] E. Yaoita, Y. Yoshida, M. Nameta, Y. Zhang, H. Fujinaka, S. Magdeldin, B. Xu, T. Yamamoto, Heparin increasing podocyte-specific gene expressions, *Nephrology* 19 (2014) 195–201, <http://dx.doi.org/10.1111/nep.12207>.
- [25] G.S. Markowitz, G.B. Appel, P.L. Fine, A.Z. Fenves, N.R. Loon, S. Jagannath, J.A. Kuhn, A.D. Dratch, V.D. D'Agati, Collapsing focal segmental glomerulosclerosis following treatment with high-dose pamidronate, *J. Am. Soc. Nephrol.* 12 (2001) 1164–1172.
- [26] L. Barisoni, W. Kriz, P. Mundel, V. D'Agati, The dysregulated podocyte phenotype: a novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy, *J. Am. Soc. Nephrol.* 10 (1999) 51–61.
- [27] J.R. Diamond, M.J. Karnovsky, Nonanticoagulant protective effect of heparin in chronic aminonucleoside nephrosis, *Ren. Physiol.* 9 (1986) 366–374.
- [28] F. Pieroni, R.S. Costa, T.M. Coimbra, Effects of glycosaminoglycans on the glomerular changes induced by adriamycin, *Res. Exp. Med.* 195 (1995) 373–379.