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Running head: FXa/IIa inhibition during kidney preservation

Abbreviations

α-SMA: α-Smooth Muscle Actin

DCD: Donors Deceased after Cardiac Death

EMT: Epithelial–Mesenchymal Transition

FBS: Fetal Bovine Serum

GAPDH: GlycerAldehyde 3-Phosphate DeHydrogenase

HAEC: Primary Human Aortic Endothelial Cells

HES: Hematoxylin - Eosin - Safran

ICAM-1: Intercellular Adhesion Molecule-1

IL: Interleukin

INRA: National Institute for Agronomic Research

iNOS: inductible Nitric Oxide Synthase

IR: Ischemia-Reperfusion

IRI: Ischemia-Reperfusion Injuries

MCP-1: Monocyte Chemoattractant Molecule-1

MMP2: Matrix MetalloProteinase-2

NEP: uninephrectomised animals

NO: Nitric Oxide

PAI-1: Plasminogen Activator Inhibitor type-1

PAR: Protease Activated Receptors

SWC3a: clone anti swine monocytes/granulocytes

TAT: Thrombin-AntiThrombin complexes

TF: Tissue Factor

TGF-β: Transforming Growth Factor-β

TNF-α: Tumor Necrosis Factor alpha

UFH: UnFractionated Heparin

UW: University of Wisconsin

VCAM-1: Vascular Cell Adhesion Molecule-1

Abstract

Coagulation is an important pathway in the pathophysiology of ischemia-reperfusion injuries. In particular, deceased after circulatory death donors undergo a no-flow period, a strong activator of coagulation. Hence, therapies influencing the coagulation cascade must be developed. We evaluated the effect of a new highly specific and effective anti-Xa/IIa molecule, with an integrated innovative antidote site (EP217609), in a porcine preclinical model mimicking injuries observed in deceased after circulatory death donor kidney transplantation. Kidneys were clamped during 60 min (warm ischemia), then flushed and preserved 24h in 4°C University of Wisconsin (UW) solution (supplemented or not). EP217609 was used in UW (UW-EP), compared to unfractionated-heparin (UW-UFH) or UW alone (UW). A mechanistic investigation was conducted *in vitro*: addition of EP217609 on endothelial cells during hypoxia at 4°C in UW inhibited thrombin generation at the 37°C reoxygenation in human plasma and reduced TNF-α, ICAM-1 and VCAM-1 mRNA cells expression. *In vivo*, function recovery was markedly improved in UW-EP group. Interestingly, levels of thrombin-antithrombin complexes (reflecting thrombin generation) were reduced 60 min after reperfusion in the UW-EP group. Three months after transplantation, lower fibrosis, epithelial-mesenchymal-transition, inflammation and leukocytes infiltration were observed in the UW-EP group. Using this new dual anticoagulant anti-Xa/IIa activity during kidney flush and preservation is protective by reducing thrombin generation at revascularization, improving early function recovery and decreasing chronic lesions. Such an easy-to-deploy clinical strategy could improve marginal graft outcome.

Background

Transplantation is currently the best therapy for end-stage renal disease. However, this success has led to an organ shortage crisis, with only one quarter of patients on waiting lists having access to organ transplantation. This crisis changed donor demography, toward an increased use of organs from marginal donors and from deceased after circulatory death donors (DCD), with significant co-morbidity factors.[1] These organs are more sensitive to the ischemia-reperfusion injury (IRI), which translates to a negative influence on graft dysfunctions. Indeed, kidneys from marginal or DCD donors are associated with higher levels of delayed graft function and primary non function.[2] This new donor demography imposes the community to investigate methods to improve graft preservation quality. In the present study, we focus on one critical process in IRI: the coagulation cascade.[3] Coagulation takes place in the vascular compartment, first target of IRI, increasing lesions such as endothelial activation and inflammation.

At the time of organ collection, the coagulation cascade is strongly activated.[3] This coagulation activation acts during organ flush (washing time after collection), somewhat less during cold storage, and mostly at the revascularization time. IRI induces vascular expression of tissue factor (TF)[4] and phosphatidylserine exposure at the outer leaflet of cell membrane.[5] Following damage to blood vessels, factor VII moves into contact with TF forming an activated complex (TF-VIIa), which activates factor X to Xa, and Xa ultimately activates factor II (prothrombin) to IIa (thrombin). The generation and signaling of Xa and IIa result in important thrombosis and inflammation.[6] Indeed, Xa and IIa cleave the protease-activated receptors (PAR) which subsequently induce the production of vascular adhesion, pro-inflammatory and pro-fibrotic molecules.[7] It was demonstrated that PAR-1 and PAR-2

activated by coagulation proteases, play a crucial role in IRI,[8,9] in association with fibrosis, tissue remodeling and inflammation.[10,11]

As a consequence, the coagulation cascade is one major target to limit IRI. Currently no inhibitors of the coagulation proteases are used in clinical settings during graft flush and preservation. However, our laboratory previously demonstrated that the use of anti-IIa (Melagatran) or anti-Xa (Fondaparinux) molecules during graft preservation could limit early and chronic lesions of transplanted kidneys.[12,13,14] In addition, another group showed that Fondaparinux reduces IRI-induced expression of pro-inflammatory cytokines/chemokines as well as neutrophils invasion in a mice kidney IR model.[15] We also demonstrated that the use of unfractionated heparin (UFH), a non-synthetic anticoagulant which exhibits pharmacokinetic and biological limitations including induction of thrombocytopenia,[16] showed less benefits compared to more specific Melagatran and Fondaparinux.[12,13,14]

In view of our previous findings, it appeared judicious to evaluate the impact of EP217609, an innovative powerful dual-action anticoagulant targeting two proteases factors IIa and Xa. EP217609 is a new anticoagulant combining, in one molecular construct, an analogue of Fondaparinux (anti-Xa) and an α-NAPAP derivative with direct thrombin inhibitory activity (anti-IIa).[17,18] The simultaneous inhibition of these two major coagulation factors (IIa and Xa), appears an interesting strategy to limit, respectively and independently, the activation of PAR-1 and PAR-2/PAR-4, key receptors of IIa and Xa at the junction of coagulation and inflammation. EP217609 targets IIa and Xa activities more rapidly and more selectively than other anticoagulant molecules, such as UFH and Fondaparinux.[17,18] Thus, EP217609 emerges as a promising new drug that is dose-dependently superior to classical (UFH, enoxaparin) and newer (Fondaparinux) drugs in preventing thrombosis.[19,20] Moreover, EP217609 includes a biotin tag with permits rapid avidin-mediated neutralization, a unique innovation in the anticoagulant arsenal, and a valuable feature for anticoagulant therapy in clinical settings.[17]

Herein, we investigated, in a preclinical pig autotransplanted kidney model, the benefits of EP217609 (hereafter designated EP) against a high level of injury induced by a period of warm ischemia prior to organ collection followed by flush and cold ischemia preservation, mimicking injuries observed in DCD situation. We used a clinically relevant and ethically appropriate porcine model, without anticoagulation before warm ischemia (in contrast of our previous study[14]), with addition of the anticoagulant EP molecule, only during the *ex vivo* flush and cold storage of the collected kidney. This porcine model offers a very high degree of correlation to the clinical situation.[21] The objective was to evaluate the action of EP therapy during kidney *ex vivo* flush and preservation, compared to UFH and no anticoagulant, on graft function recovery and chronic injuries (3 months post-transplantation).

Methods

In vitro cellular model

Primary human aortic endothelial cells (HAEC) were cultured on 1% gelatin (Sigma, France) in Medium 200 supplemented with 2% Low Serum Growth Factor and 10% fetal bovine serum (FBS) (Invitrogen, France) in a humidified atmosphere at 5% CO_2 and 37°C. Cells were used at passage 4. At confluent culture and after synchronisation, cells were submitted to cold hypoxia in UW (also referred to as *in vitro* cold "ischemia-like" period): incubation in 0% O_2, 5% CO_2, and 95% N_2 atmosphere (Bactal 2 gaz, Air Liquide, France) in a hermetic chamber at 4°C, during 24h, in UW preservation solution (University of Wisconsin solution; Bristol-Meyers-Squibb, France) +/- EP (16.3 mg/L = 5.77 μM). At the end of this cold hypoxia period, a reoxygenation phase (also referred to as *in vitro* "reperfusion-like" period) was applied at 37°C in a humidified atmosphere 20% O_2, 5% CO_2, and 75% N_2: replacing UW by Medium 200 + 2% FBS for 2h or replacing by "activated plasma". In the objective to obtain "activated plasma" without clot formation, we first added 1/50 of a reptilase solution (20 Batroxobin Units/ml, Cryopep, France) in normal human, low-platelets plasma previously collected on sodium citrate 3.2% (Cryopep, France), incubated at 37°C during 5 minutes, then 15 minutes at 4°C, and the clot was removed (Sigma-Aldrich, France). Finally the plasma was "activated" with 20 mM $CaCl_2$ at the reoxygenation time. After this *in vitro* cold hypoxia ± reoxygenation sequence, HAEC cells were collected for mRNA, western blot analysis and flow cytometry, and plasmas were collected for quantification of thrombin generation.

Flow cytometry

HAEC cells were stained with membrane antibody anti-TF (ab88387; Abcam, France) and Annexin V (556547; Bd-Pharmingen, France) diluted in phosphate-buffered saline containing

2% FBS and analysed. Appropriate isotype control was used for the TF phenotyping. Cells were analysed by Fluorescence Assisted Cell Sorting Canto II™ and FacsDiva™ software (BD, Franklin Lakes, NJ) and data were analysed using FlowJo™ software (version 7.5.5 Treestar, Ashland, OR).

Quantification of thrombin generation

Thrombin generation rate was measured in the plasma on HAEC at the time of *in vitro* reoxygenation period. The reaction is based on a fluorescence substrate cleavage by the neo-synthetized plasma thrombin in the presence of $CaCl_2$ (FluCa Kit, Stago). Fluorescence was detected on a Fluoroskan Ascent (Thermo) with the Thrombinoscope® software. The generation curve showed typical phases: the lag time before thrombin generation (min), the peak of the maximal thrombin measurement (nM), the time to peak to obtain the maximum thrombin concentration (min), the area under the curve (AUC) of the total thrombin generation (nmol/L.min^{-1}), and the start tail corresponding to the end of IIa generation (min).

In vivo model

All animal experiments conformed to the guidelines for the Care and Use of Laboratory Animals and approved by French Poitou-Charentes ethical comity of animal experimentation (protocol number CE2012-4) and National Institute for Agronomic Research (INRA). We used 3 months old Large White pigs weighing 40 ± 4 kg (IBiSA, INRA Magneraud, France). Briefly, as previously described,[22] after anesthesia the left kidney was approached through a midline abdominal incision. The left renal vascular pedicle and the ureter were atraumatically dissected, and warm ischemia was induced by renal pedicle clamping during 60 min. Then the kidney was removed and immediately flushed with 4°C University of Wisconsin preservation

solution (UW; Bristol-Myers-Squibb, France), supplemented or not with UFH or EP217609 and stored at 4°C for 24h.

The left kidney was collected after 60 min warm ischemia induced by arterial clamping, then cold-flushed and preserved with UW solution, supplemented or not, during 24 hours at 4°C under static condition. At the end of the preservation, the contralateral right kidney was removed and the preserved left kidney was transplanted in the same animal by heterotopic autotransplantation *via* the midline incision. End-to-side aorta and inferior vena cava anastomoses, just above the iliac bifurcation, were performed. Ureteroneocystostomy was performed.

Four groups were studied: Control (NEP) : this group was uninephrectomised (only the left kidney was left in place) to mimick the nephron mass reduction induced by transplantation (n=6), UW: Left kidney was submitted to 60 min warm ischemia, then collected and flushed + preserved with UW alone (n=6), all animals in this group developed Primary Non-Function within the first week after transplantation; UW-UFH: Left kidney was submitted to 60 min warm ischemia, then collected and flushed + preserved with UW+UFH (5,000 UI *per* L UW) (n=6); UW-EP: Left kidney was submitted to 60 min warm ischemia, then collected and flushed + preserved with UW+ EP217609 (16.3 mg *per* L UW = 5.77 µM) (n=5), one of the animal was excluded because or surgery problem. None of the anticoagulants induced bleeding in any animal.

Renal function evaluation

Plasma creatinine was measured with a Modular bioanalyser (Roche-Diagnostics, France), using the modified compensated Jaffé technique. Diuresis was evaluated by urine production after transplantation.

Morphological study

Biopsy samples from corticomedullary kidney collected at day 14 or 3 months after transplantation were fixed with 4% formalin and paraffin-embedded. For immunohistochemistry, we used the following antibodies: anti-vimentin (Cell-Marque, USA), anti α-smooth muscle actin (α-SMA, Sigma-aldrich, France) and anti-SWC3a reacting with monocytes/macrophages (553640; BD-Pharmingen, France), positive staining was revealed using Diaminobenzidine colorimetric reaction (DAKO, France). Vimentin staining quantification was performed by counting the number of vimentin positive tubes *per* field ; α-SMA and Picro-Sirius staining quantification was performed by quantifying the percentage of staining *per* field (Visilog 6.9 software); and evaluation for SWC3a staining was performed by counting the number of positive cells *per* field. We evaluated 10 fields *per* tissue sample (magnification x100 to Picro-Sirius, α-SMA and Vimentin, and x200 to leukocytes infiltrate and SWC3a).

Soluble proteins quantification

Thrombin-antithrombin complexes (TAT) (Antibodies-Online, France) were assessed in porcine plasma using ELISA kit according to the manufacturer's instructions.

Real-Time Quantitative-PCR

Total RNAs from porcine renal tissue and HAEC were extracted on columns (Macherey-Nagel, France). Genomic DNA was removed using DNA-free kit (Applied-Biosystems, France) and first-strand reverse transcription (Applied-Biosystems) was performed. Real-Time PCR assays were carried out on a RotorGene Q (Qiagen, France) following the manufacturer's recommendations. DNA primers were designed using OligoPerfect™ (Invitrogen), QuantPrim (Universität Potsdam, Max-Planck-Gesellschaft) and OligoAnalyser

(Integrated DNA Technologies Inc) with the sequences detailed in supplementary Tables S1 and S2. For *in vitro* HAEC cells transcriptome analysis, mRNA expression levels, expressed as "relative to expression in control cells", were determined with the Pfaffl method (expressed as Relative Fold Change), using the most stable genes as internal controls: the ribosomal L19 and S19, chosen after their stability between groups was ascertained with the geNorm algorithm. For porcine transcriptome analysis, mRNA expressions, expressed as "relative to expression in healthy kidney", were determined with the Pfaffl method (expressed as Relative Fold Change), using β-actin, RPLPO and L19 genes as stable internal controls.

Western blot procedure

Western blot analyses were performed using specific antibodies against: TF (ab88387; Abcam, France), inductible Nitric Oxide Synthase (iNOS; 610431; BD-Transduction, France), Transforming Growth Factor-β (TGF-β; sc-146; Santa-Cruz, France), Matrix Metalloproteinase-2 (MMP2; sc-10736; Santa Cruz, France), Interleukine-8 (IL-8; ab18672; Abcam, France), Monocyte Chemoattractive Protein-1 (MCP-1; 5225-100; Biovision) and loading control: Glyceraldehyde 3-Phosphate DeHydrogenase (GAPDH; CB1001; Sigma Aldrich, France) or β-actin (A1978; Sigma Aldrich, France).

Statistical methods

For Figure 1D and 2B, results are expressed in mean ± SD. For Figure 2A and 2B, statistical analyses were performed according to two-factors ANOVA and Bonferroni post-test (GraphPad software). For other data, results are expressed as Scatter plot with median and Interquartile range, and statistical analysis were performed with Kruskall-Wallis test and Dunn's post-test, except for Figure 1B, E1, and 1F for which statistical analysis were performed as Mann-Whitney test (NCSS software).

Results

EP217609 treatment during *in vitro* cold hypoxia period inhibits thrombin generation on an "ischemic-like" procoagulant endothelial cell surface

In order to assess the effects of anti-Xa/IIa treatment on the endothelium, we developed an *in vitro* model of cold hypoxia + reoxygenation 37°C with primary HAEC. Under these conditions, after *in vitro* reoxygenation period, there was a significant up-regulation of cellular production of TF (measured by western blot after 15 min and 1 h of reoxygenation) and a significant increase of TF and phosphatidylserine on the cell membrane (measured by flow cytometry) (Figure 1A and 1B). These results highlighted that "ischemia-reperfusion" sequence induces a procoagulant endothelial cell surface, favoring coagulation proteases activation. In *in vitro* cold hypoxia period in UW alone condition, thrombin generation was observed at reoxygenation time in human "activated" plasma, with a peak at 15 min of reoxygenation period. However, the use of EP (16.3 mg/L), during *in vitro* cold hypoxia period, totally inhibited thrombin generation at reoxygenation time in "activated" plasma (Figure 1D-E). In parallel experiments carried out in this *in vitro* model, treatment with EP during cold hypoxia period was able to significantly reduce Tumor Necrosis Factor alpha (TNFα), Intercellular Adhesion Molecule-1 (ICAM1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) mRNA expression at 2 hour of reoxygenation period (Figure 1F).

In vivo, EP217609 treatment improves renal function after IR and reduces systemic TAT levels

In order to assess renal function after autotransplantation, we recorded diuresis recovery and plasma creatinine level during three months after transplantation. The first transplanted group to recover diuresis was the EP group at day 2; at day 3, only half of the animals in the UFH

group resumed urine production (figure 2A). All animals of the UW group developed primary non function, defined by the absence of urine production at day seven after reperfusion; they were not included in the long-term post-transplantation evaluation. Area under the curve analysis of plasma creatinine from day 0 to day 7 indicated a significantly better renal function in the anticoagulant treated groups as compared to UW group (figure 2C). The treatment with EP during conservation reduced significantly the plasma creatinine at days 14, 30 and 90 after transplantation, as compared to UFH treatment (figure 2D, 2E and 2F). At 3 months, creatininemia in the EP group was identical to pre-transplantation baseline, when animals had two kidneys (mean of creatinemia: D0 = 84 µmol/L, M3 = 88 µmol/L).

Systemic generation of thrombin after 60 min kidney reperfusion was evaluated by TAT plasma level measurement, and showed less TAT levels in both anticoagulant treated groups compared to UW alone (figure 3).

EP217609 treatment reduces graft chronic injuries; Epithelial to Mesenchymal Transition (EMT) and fibrosis

We quantified histological interstitial fibrosis with red Sirius staining (collagen fibers I and III staining), and EMT with two markers: α-SMA and vimentin. In the renal tissue at three months, there was significantly less fibrosis (Sirius red staining) in the EP group than in the UFH group (figure 4B). α-SMA was significantly less expressed in the EP and NEP groups as compared to the UFH group (figure 4C). The number of tubules expressing vimentin was lower in the EP and NEP groups than in the UFH group (figure 4D).

At 3 months after transplantation, the profibrotic marker Plasminogen Activator Inhibitor type-1 (PAI-1) mRNA was upregulated in the UFH treated group, as compared to NEP and EP groups (figure 5A). Kidney cortex expression of MMP2 protein was significantly lower in

the EP treated group compared to NEP and UFH groups (figure 5B). We observed no difference between groups for TGF-β protein expression (figure 5C).

EP217609 treatment reduces the pro-inflammatory environment and leukocyte infiltration

In order to evaluate the effect of EP treatment on chronic injuries 3 months after transplantation, we measured the expression of inflammatory markers mRNA and proteins. mRNA and protein expression of MCP-1 appeared lower ($p=0.08$) in the EP treated group compared to UFH group (figure 5D-E). mRNA expression of IL-1β was significantly higher in the UFH group compared to NEP and EP groups (figure 5F). Inducible NOS (iNOS) protein expression was significantly lower in the EP group when compared to the UFH group (figure 5G). mRNA expression of IL-8 was significantly higher in the UFH treated group compared to NEP group (figure 5H). A similar profile was obtained for IL-8 protein expression, although the difference between UFH and EP groups was less pronounced (figure 5I).

In order to evaluate the leukocytes infiltration in the graft, we performed a histological study on kidneys 14 days and 3 months after transplantation. Leukocytes infiltration score established on Hematoxylin-Eosin-Safran (HES) staining showed that, at day 14 after transplantation, the UFH group exhibited the most important level of infiltrated cells (figure 6B). At 3 months the treatment with EP significantly reduced leukocytes infiltration compared to UFH treatment (Figure 6A-C). We quantified by immunohistochemistry the number of monocytes/macrophages infiltrates in the graft using SWC3a labeling, and observed a significantly higher number of these cells in the UFH group as compared to NEP and EP groups (figure 6D-E).

Discussion

In this study, we evaluated the anti-Xa/anti-IIa anticoagulant EP217609 molecule ability to limit ischemia-reperfusion injuries both in an *in vitro* model and in a preclinical porcine kidney transplantation model, as compared to UFH or no anticoagulant. UFH, here used as the control molecule, is currently used in clinic. As EP217609, it has a dual anti IIa and anti Xa activity, whereas Melagatran and Fondaparinux target only one factor (respectively factor IIa and factor Xa).

Using an *in vitro* model of human endothelial cells, we demonstrated that a cold "ischemia-like" period (experimentally mimicked by hypoxia UW incubation at 4°C during 24h) induced, after the "reperfusion-like" period (experimentally mimicked by reoxygenation at 37°C in 20% O_2), an increase of TF protein cellular expression as well as TF and phosphatidylserine expression on the endothelial membrane. These results confirm that ischemia-reperfusion (IR) induces a pro-coagulant state of endothelial cell surface, capable of activating coagulation proteases and the generation of thrombin. This observation led us to develop an *in vitro* HAEC model with a cold "ischemia-like" period, followed by a "reperfusion-like" phase in normal "activated" plasma in order to determine the effect of EP on "normal" plasma at the "reperfusion-like" time. Interestingly, in this *in vitro* model, the addition of EP during the 24h cold "ischemia-like" period inhibited thrombin generation at "reperfusion-like" time in plasma. During the transplantation process, the activation of coagulation proteases, particularly factor Xa and thrombin, is an important contributor to the negative effects of coagulation, inducing inflammation and fibrosis *via* protease-activated receptor activation.[7,10,11] In this context, factor Xa and thrombin induce the expression of pro-inflammatory markers and adhesion molecules, particularly in endothelial cells and blood mononuclear cells.[23,24] We show in our *in vitro* model of cold "ischemia-like" + "reperfusion-like" sequence that treatment of endothelial cells with EP217609 during cold "ischemia-like"

period permits a significant decrease of endothelial activation by reducing TNFα mRNA expression, as well as the expression adhesion molecules ICAM-1 and VCAM-1 mRNA at the "reperfusion-like" period.

TAT complexes, issued from the neutralization of thrombin by antithrombin III, have been used as a surrogate marker for thrombin generation.[25] A revascularisation time, an increase of TAT was demonstrated, with a significant correlation between plasma level of TAT and ischemia degree as well as with the duration of surgery.[25] Herein, *in vivo*, we observed a decrease of TAT formation at 60 min post-reperfusion when the kidney was preserved with anticoagulants. Since a direct link between thrombin activity and pro-inflammatory response has been established in IR context, in mice[26] and rat,[27] our results further suggest that EP217609-dependent "thrombin" inhibition may limit inflammation.

Here, we designed a preclinical model mimicking DCD donation with an anticoagulation protocol more applicable to clinics than our previous Fondaparinux study. Indeed, in this previous study, Fondaparinux was injected before warm ischemia and added in the UW preservation solution,[14] whereas here EP217609 was added only in the UW preservation solution. This procedure avoids intravenous administration and reduces required amounts of compound. Moreover, UW control groups in these two studies are not comparable. In the Fondaparinux study,[14] animals of the UW group received an UFH injection before warm ischemia, whereas, in order to conform to clinical constraints, this was not the case here. Of note, a previous study with our porcine model suggested that pre-warm ischemia UFH injection could improve organ recovery, with lower PNF.[28] Herein, treatment with EP217609 during kidney flush + cold storage improved renal function recovery, as compared to UW group, in association with the reduction of thrombin generation (no TAT detectable) at reperfusion. In the EP group as compared to UW-UFH group, the improved early graft recovery is associated with a better renal function at days 14, 30 and 90 post-transplantation.

Better graft function was associated with a reduction, at 3 months, of fibrosis (Sirius red immunohistological staining), as well as EMT (defined by markers α-SMA and vimentin in immunohistological analysis), a well described pathway leading to interstitial fibrosis.[29] The later observations are strengthened by protein and mRNA expression analyses, as indeed EP reduced the expression of PAI-1 mRNA, a mediator of fibrosis,[30] and metalloproteinase MMP-2 protein, implicated in EMT and fibrosis.[29] There was no difference in TGF-β protein expression, possibly due to analysis timing (3 months post-transplantation) in relation to the kinetics of fibrosis development, suggesting that TGF-β-dependent mechanism of fibrosis initiation could be terminated.

Inflammation is known to be intimately linked to fibrosis development and chronic rejection. We thus focused on the expression of chemoattractant cytokine (IL-8 and MCP1) and pro-inflammatory IL-1β and iNOS, all linked to leukocyte activation. We showed that chemoattractant MCP-1 expression was reduced in the EP group as compared to UFH. IL-8 expression was upregulated in the UFH group as compared to NEP. Expression of pro-inflammatory IL-1β and iNOS markers was significantly lower in the EP group as compared to the UFH group. Such benefits of EP treatment were associated with a limitation of immune cells infiltration. Indeed, at day 14 and day 90 after transplantation, histological study with HES staining and SWC3a immunostaining showed significantly lower leukocytes infiltrates in the EP group compared to the UFH group.

Our findings clearly demonstrate that EP is more efficient in graft protection than UFH, a standard anticoagulant. This may be due to the fact that UFH cannot prevent the thrombin rebound,[31] a phenomenon through which thrombin trapped by the clot is later released in the blood stream, possibly to exert his effects, leading to inflammation and endothelial activation.

EP217609 exhibits an unprecedented pharmacologic profile with high bioavailability, long plasma half-life (20 h) (to be compared to 90 min for UFH), and potent antithrombotic

activity in animals, without the complications of thrombin rebound.[17,18] Our results showed that EP217609 was effective as early as 60 min after kidney reperfusion (i.e. 24h after application) suggesting a long half-life of this molecule at 4°C (> 24 h). Compared to heparin, EP217609 displays a similar antithrombotic effect, though at a much lower dose, but it is devoid of activity on platelets (no induced thrombocytopenia) and displays predictable and highly reproducible pharmacokinetics. Studies have shown that EP217609 inhibits thrombin with rapid kinetics ($k_{on} > 10^7$ $M^{-1}s^{-1}$), high affinity (K_I = 30-40 pM), and more than 1,000-fold selectivity over other coagulation and fibrinolytic protease targets, thus comparing favorably with the best known direct thrombin inhibitors. EP217609 binds to antithrombin with high affinity (K_D = 30 nM), 2-fold stronger than Fondaparinux, and activates serpin to rapidly (k_{ass} ~ 10^6 $M^{-1}s^{-1}$) and selectively (>20-fold) inhibits factor Xa.[18] The dual inhibitory moieties of EP217609 act largely independently with only modest linkage effects of ligand occupancy of one inhibitor moiety over the potency of the other (~ 5-fold). In contrast, avidin binding effectively neutralized the potency of both inhibitor moieties (20- to 100-fold).[18] Oslon and coworkers demonstrated the superior anticoagulant efficacy and rapid avidin neutralizability of EP217609 compared with anticoagulants that target thrombin or factor Xa alone.[17] This molecule thus presents an improved activity profile, as well as the added safety provided by its antidote site compared to other anticoagulants, as Fondaparinux.

In summary, our results demonstrate that this anticoagulant with a new specific dual activity (anti Xa/IIa), can be used, easily and cost-effectively, during kidney graft flush + preservation to protect the graft against the deleterious consequences of IR, by i) reducing thrombin generation at reperfusion, ii) improving early function recovery, and iii) decreasing chronic lesions (fibrosis, EMT and inflammation). Hence, specific therapy with EP217609 appears a valuable tool to improve graft quality in clinics: it is easy to deploy (supplementation of

existing preservation solutions), and the existence of a rapid antidote increases its safety of use compared to other anticoagulants.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Figure legends:

Figure 1: Evaluation of thrombin generation following *in vitro* "hypoxia 4°C ± EP217609 - reoxygenation 37°C" sequence on human endothelial cells (HAEC).

A: Quantification by western blot of Tissue Factor (TF) protein expressed on HAEC cells surface after 24h hypoxia 4°C in UW solution (Hypoxia UW) and reoxygenation 37°C in culture medium during 15 min (Hypoxia UW + reox 15min) and 1h (Hypoxia UW + reox 1h) *versus* control healthy cells (Ctl cells). Results are expressed as Scatter plot with median and Interquartile range. Statistical significance (* $p<0.05$) was calculated using Kruskall-Wallis test and Dunn's post-test (N=3). B: Quantification of TF and phosphatidylserine (PS, detected by Annexin V labeling) expression by flow cytometry, on HAEC cells surface after 24h hypoxia 4°C in UW solution and reoxygenation 37°C in culture medium during 2h (Hypoxia UW + reox), compared to non-stimulated healthy cells (Ctl cells). Results are expressed as Scatter plot with median and Interquartile range. C: Flow cytometry representative histograms of TF and Annexin V expression by flow cytometry. Statistical significance (* $p<0.05$) was calculated using Mann-Whitney test (N=3). D: Quantification (mean ± SD) of thrombin generation (nmol/L) by HAEC cells at the reoxygenation time in normal human "activated" plasma (reoxygenation time following a hypoxia sequence at 4°C in UW during 24h ± EP217609; labelled Hypoxia UW or Hypoxia UW-EP). E: Area under the curve (AUC) of the amount of thrombin generated (nmol/L.min) related to Figure 1D. Results are expressed as Scatter plot with median and Interquartile range. Statistical significance (* $p<0.05$) was calculated using Mann-Whitney test (N=3). F: Expression of mRNA TNFα, ICAM-1 and VCAM-1 on HAEC cells at the end of 2h of reoxygenation (labelled: reox 2h) after hypoxia in UW 4°C with EP (Hypoxia UW-EP) or without (Hypoxia UW) then, versus control healthy cells (Ctl). Expression is shown as relative fold change to ctl cells. Results are expressed as

Scatter plot with median and Interquartile range. Statistical significance (* $p<0.05$) was calculated using Mann-Whitney test (N=3).

Figure 2: Effect of EP217609 (EP) on renal function outcome after kidney autotransplantation.

Experimental groups: UW alone (UW, n=6), UW + UFH 5,000 UI/L (UW-UFH, n=6), UW + EP217609 16.3 mg/L (UW-EP, n=5) and uninephrectomized group (NEP, n=6). A: Percentage of animals with positive diuresis recovery on the first four days after kidney transplantation following preservation in UW, UW-UFH or UW-EP compared to non transplanted NEP. B: Mean ± SD of post-reperfusion plasma creatinine levels (µmol/L) quantified from day 0 to days 90 after kidney transplantation. Statistical analysis (Figure 2A, 2B) were performed with two factors ANOVA and Bonferroni post-test (Figure 2A' and 2B'). C: Post-reperfusion plasma creatinine levels expressed in area under the curve from day 0 to day 7 post-transplantation (µmol/L.day). Post-reperfusion plasma creatinine levels (µmol/L) quantified at day 14 (D), day 30 (E) and day 90 (F) after kidney transplantation. Results are expressed as median with Interquartile range. Statistical significance ($p<0.05$) were calculated using Kruskall Wallis test and Dunn's post-test, * $p<0.05$ versus NEP, £ $p<0.05$ versus UW-UFH, $ $p<0.05$ versus UW-EP, # $p<0.05$ versus UW.

Figure 3: Effect of EP217609 (EP) on early blood coagulation activation after kidney transplantation.

Quantification (pg/mL) of plasma TAT (Thrombin-AntiThrombin complexes), reflecting thrombin IIa generation, at 60 min after kidney transplantation (n=5). Results are expressed as Scatter plot with median and Interquartile range. Statistical significance (* $p<0.05$) was calculated using Kruskall-Wallis test and Dunn's post-test.

Figure 4: Effect of EP217609 (EP) on histological fibrosis and EMT at 3 months after kidney transplantation.

Left panel; A: Representative pictures of each group, fibrosis (Sirius red staining) x100, α-SMA x100 and vimentin x100 (arrows indicate positive tubules). Right panel; B: fibrosis expressed as percentage of Sirius red staining *per* field. C: percentage of α-SMA staining *per* field. D: number of vimentin positive tubules *per* field. Results are expressed as Scatter plot with median and Interquartile range (n=5-6). Statistical significance ($p<0.05$) was calculated using Kruskall-Wallis test and Dunn's post-test, * $p<0.05$ versus NEP, £ $p<0.05$ versus UW-UFH, $ $p<0.05$ versus UW-EP.

Figure 5: Effect of EP217609 (EP) on cortical fibrosis and EMT markers expression at 3 months after kidney transplantation.

A, D, F, H: mRNA quantification (relative fold-change to day 0 before transplantation) of respectively PAI-1, MCP-1, IL-1β and IL-8 in the cortex 3 months after kidney transplantation (n=5-6, expression in % of NEP). B, C, E, G, I: Expression of proteins MMP2, TGF-β, MCP-1, iNOS and IL-8 in the cortex 3 months after kidney transplantation (n=5, expression in % to NEP), quantified as ratio to control protein (GAPDH or β actin). Blots are shown above each histogram. Results are expressed as Scatter plot with median and Interquartile range. Statistical significance ($p<0.05$) was calculated using Kruskall-Wallis test and Dunn's post-test, * $p<0.05$ versus NEP, £ $p<0.05$ versus UW-UFH, $ $p<0.05$ versus UW-EP (n=5).

Figure 6: Analysis of leukocytes infiltration in renal tissue, 14 days and 3 months after kidney transplantation.

A: HES staining (x200) representative pictures of each group for the histological evaluation of leukocyte infiltration in kidneys 3 months after transplantation (J90). B, C: Tissue leukocytes infiltrate scoring, on HES staining, at 14 and 90 days after kidney transplantation. Scores: 0= no infiltrate, 1= weak localized infiltrate, 2= weak diffused infiltrate, 3= intense localized infiltrate, 4= intense diffused infiltrate, and 5= intense diffused infiltrate with tubulitis. D: representative pictures (x200) of SWC3a labelling (anti monocyte/macrophage) for each group in kidneys 3 months after transplantation (J90). E: Evaluation of SWC3a positive cells infiltration in kidney 3 months after kidney transplantation. Immune SWC3a cells infiltration was determined by quantification of number of SWC3a cells *per* field. Results are expressed as Scatter plot with median and Interquartile range (n=5-6). Statistical significance (* $p<0.05$) was calculated using non-parametric Kruskall-Wallis test and Dunn's post-test.

Figure 1

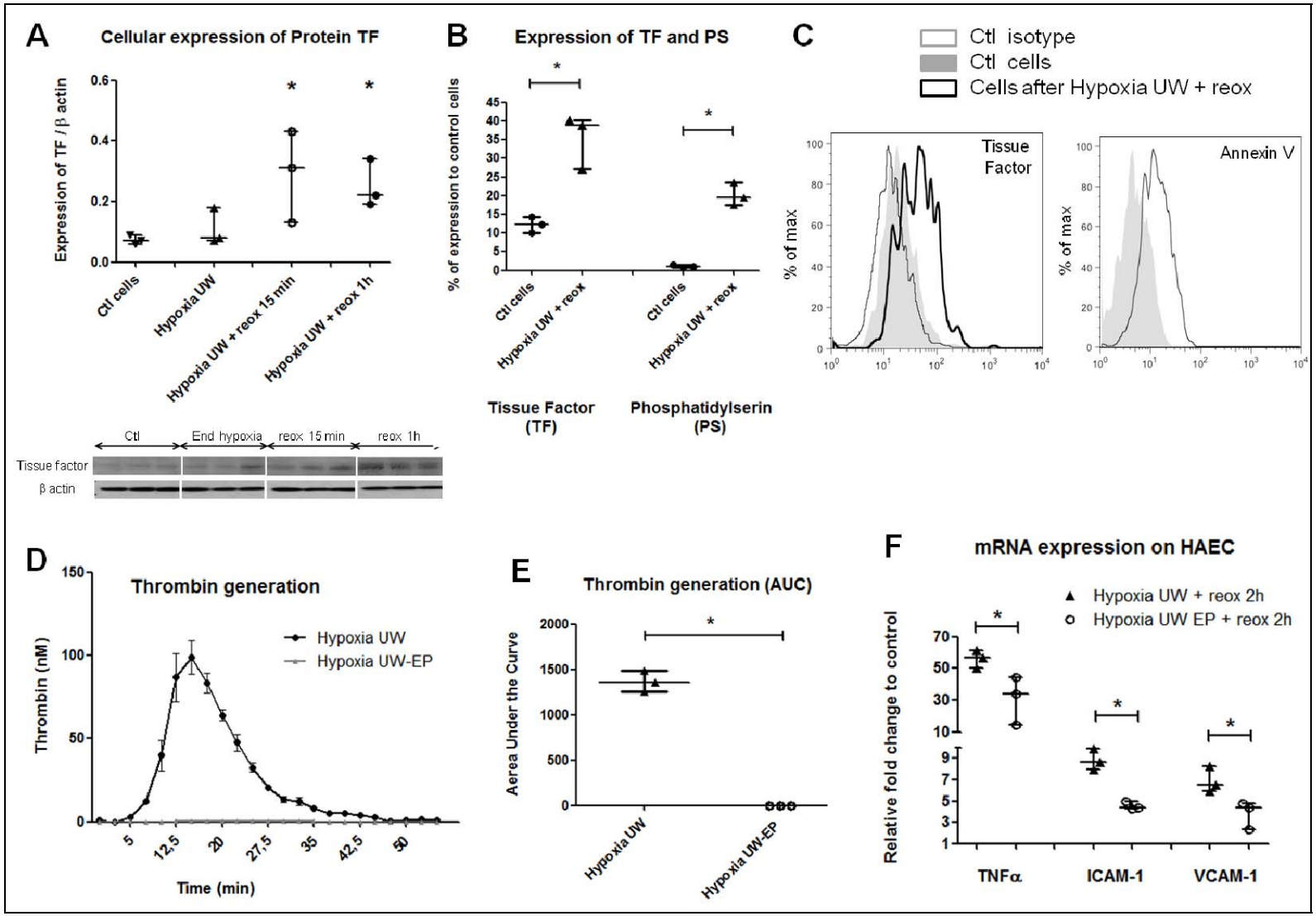
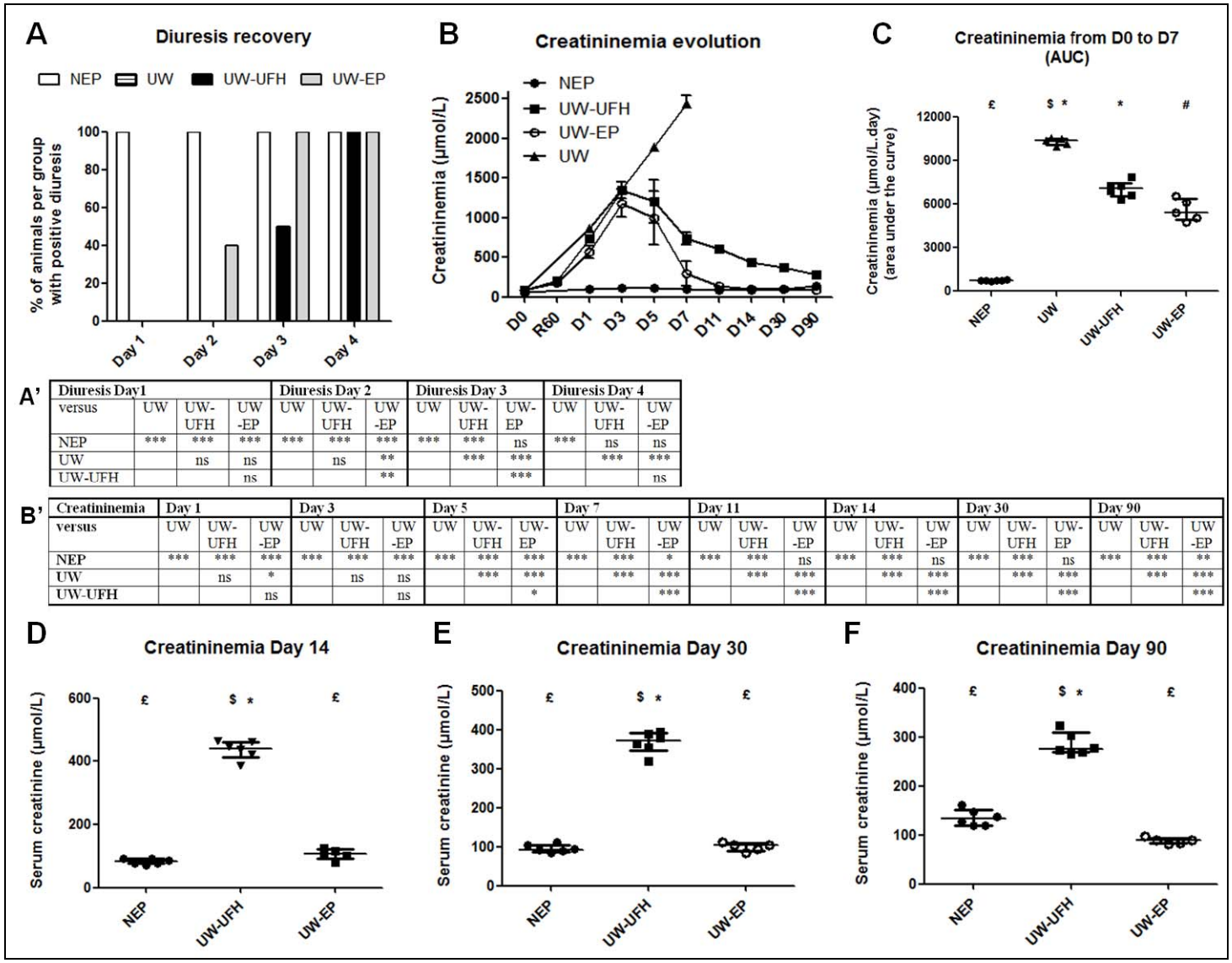

A
Cellular expression of Protein TF
Expression of TF / β actin
Ctl cells
Hypoxia UW
Hypoxia UW + reox 15 min
Hypoxia UW + reox 1h
Ctl
End hypoxia
reox 15 min
reox 1h
Tissue factor
β actin
B
Expression of TF and PS
% of expression to control cells
Ctl cells
Hypoxia UW + reox
Tissue Factor (TF)
Phosphatidylserin (PS)
C
Ctl isotype
Ctl cells
Cells after Hypoxia UW + reox
Tissue Factor
Annexin V
% of max
D
Thrombin generation
Hypoxia UW
Hypoxia UW-EP
Thrombin (nM)
Time (min)
E
Thrombin generation (AUC)
Aerea Under the Curve
Hypoxia UW
Hypoxia UW-EP
F
mRNA expression on HAEC
Hypoxia UW + reox 2h
Hypoxia UW EP + reox 2h
Relative fold change to control
TNFα
ICAM-1
VCAM-1

Figure 2



A'

Diuresis Day1				Diuresis Day 2			Diuresis Day 3			Diuresis Day 4		
versus	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP
NEP	***	***	***	***	***	***	***	***	ns	***	ns	ns
UW		ns	ns		ns	**		***	***		***	***
UW-UFH			ns			**			***			ns

B'

Creatininemia	Day 1			Day 3			Day 5			Day 7			Day 11			Day 14			Day 30			Day 90		
versus	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP	UW	UW-UFH	UW-EP
NEP	***	***	***	***	***	***	***	***	***	***	***	*	***	***	ns	***	***	ns	***	***	ns	***	***	**
UW		ns	*		ns	ns		***	***		***	***		***	***		***	***		***	***		***	***
UW-UFH			ns			ns			*			***			***			***			***			***

Figure 3

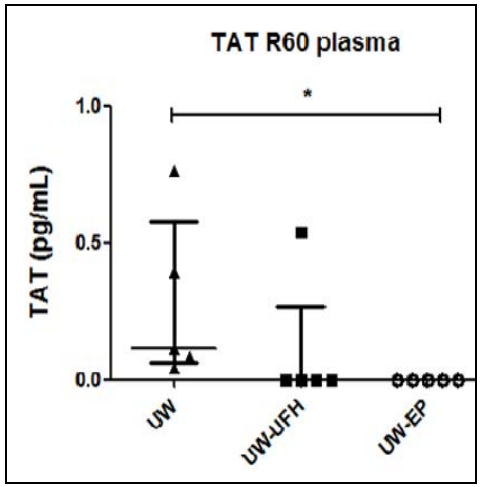


Figure 4

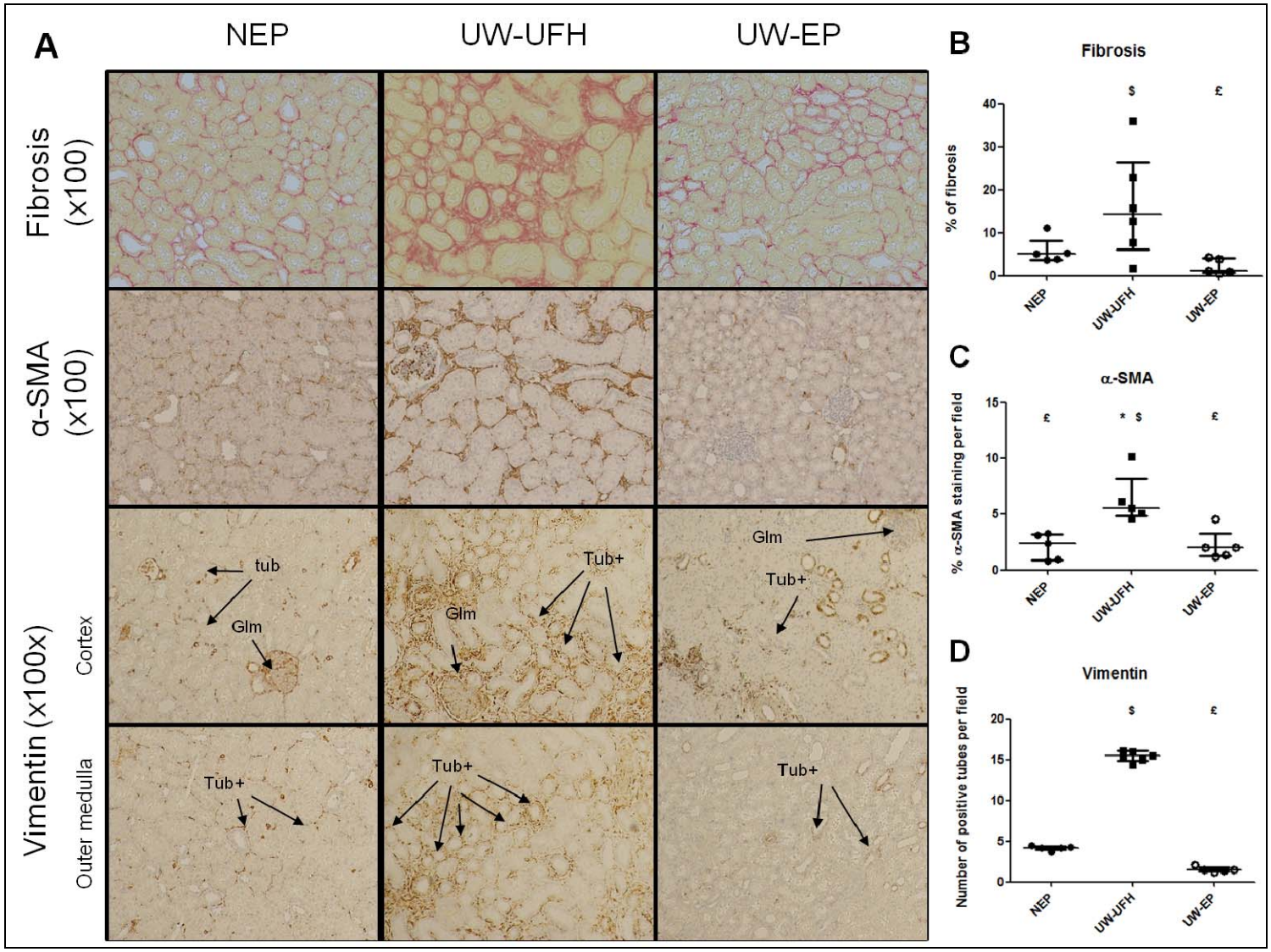

A
NEP
UW-UFH
UW-EP
Fibrosis (x100)
α-SMA (x100)
Vimentin (x100x)
Cortex
Outer medulla
tub
Glm
Tub+
B
Fibrosis
% of fibrosis
C
α-SMA
% α-SMA staining per field
D
Vimentin
Number of positive tubes per field

Figure 5

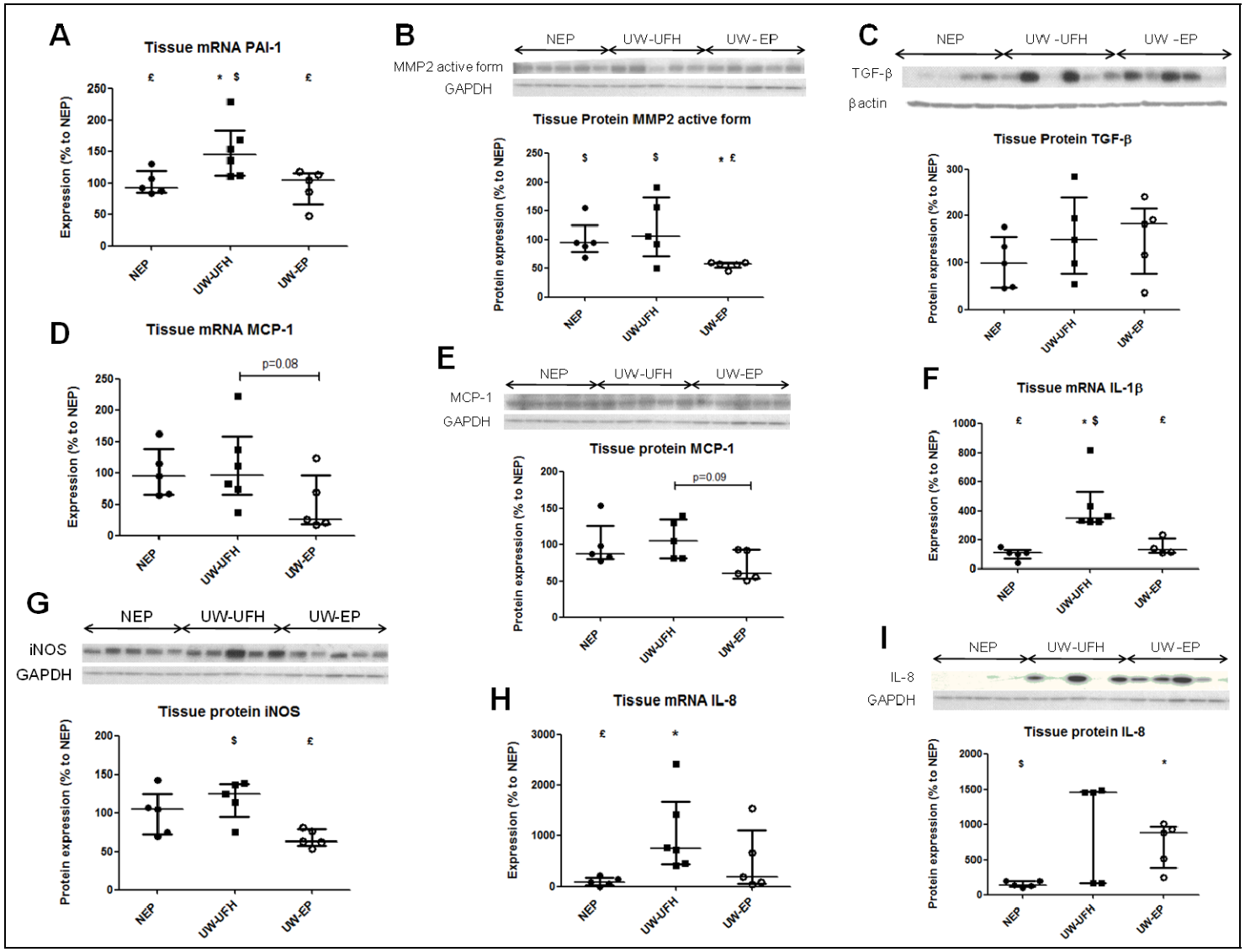
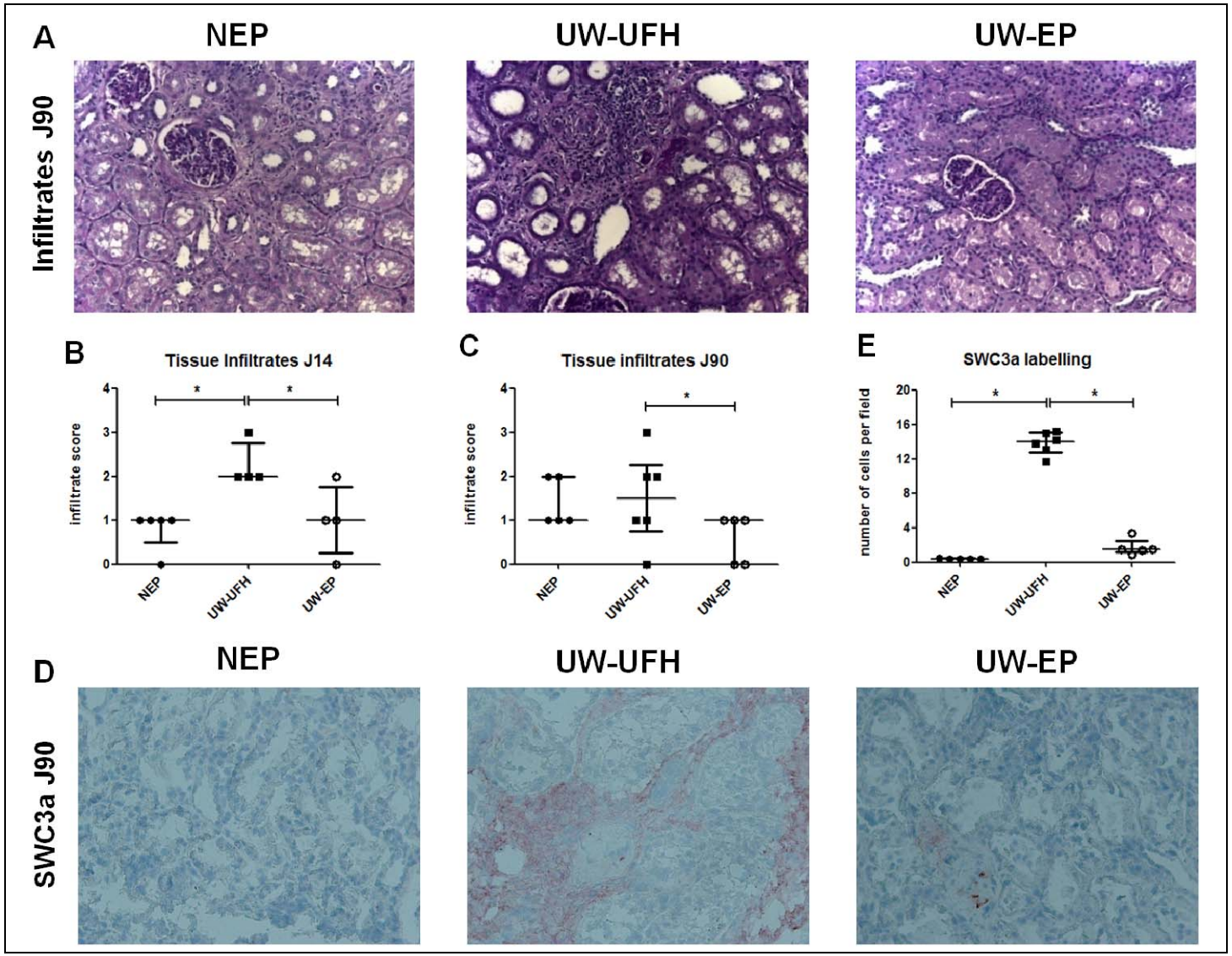

A
Tissue mRNA PAI-1
Expression (% to NEP)
NEP
UW-UFH
UW-EP
B
NEP
UW-UFH
UW-EP
MMP2 active form
GAPDH
Tissue Protein MMP2 active form
Protein expression (% to NEP)
C
NEP
UW-UFH
UW-EP
TGF-β
β actin
Tissue Protein TGF-β
Protein expression (% to NEP)
D
Tissue mRNA MCP-1
p=0.08
Expression (% to NEP)
E
NEP
UW-UFH
UW-EP
MCP-1
GAPDH
Tissue protein MCP-1
p=0.09
Protein expression (% to NEP)
F
Tissue mRNA IL-1β
Expression (% to NEP)
G
NEP
UW-UFH
UW-EP
iNOS
GAPDH
Tissue protein iNOS
Protein expression (% to NEP)
H
Tissue mRNA IL-8
Expression (% to NEP)
I
NEP
UW-UFH
UW-EP
IL-8
GAPDH
Tissue protein IL-8
Protein expression (% to NEP)

Figure 6


A
NEP
UW-UFH
UW-EP
Infiltrates J90
B
Tissue Infiltrates J14
infiltrate score
NEP
UW-UFH
UW-EP
C
Tissue infiltrates J90
infiltrate score
NEP
UW-UFH
UW-EP
E
SWC3a labelling
number of cells per field
NEP
UW-UFH
UW-EP
D
NEP
UW-UFH
UW-EP
SWC3a J90

Supporting Information (description):

Supplementary Table S1: Primer sequences for RT-PCR analysis in HAEC cells

Gene	**Forward**	**Reverse**
L19	CAGGAAGAGGACTTGGAGCTAC	GCTATCATCCAGCCGTTTCTCTA
RPLPO	CAGGGCGACCTGGAAGT	TCTGCTTGGAGCCCACA
S19	GACGTGAACCAGCAGGAGTT	CTTGACGGTATCCACCCATT
S5	CTCTTTGGGAAGTGGAGCAC	TGCACTGTGAGGCAGGTACT
TNFα	CCCAGGGACCTCTCTCTAATCA	GCTACAGGCTTGTCACTCGG
VCAM-1	AAGATGGTCGTGATCCTTGG	TAGATTCTGGGGTGGTCTCG
ICAM-1	TGCTATTCAAACTGCCCTGA	CACTGGCTGCCAAGAGG

Supplementary Table S2: Primer sequences for RT-PCR analysis in porcine kidney

Gene	**Forward**	**Reverse**
L19	AATCGCCAACGCCAACTC	CAGCCCATCTTTGATCAGCTT
RPLPO	AGAAACTGTTGCCTCACATCC	CCTTATTGGCCAGCAGCA
β actin	GATCGTGCGGGACATCAAG	GCCATCTCCTGCTCGAAGTC
SDHA	GAGTTCGTGCAGTTCCACCCTA	CCTCTCACCCTGGCTGTTGATA
IL-1β	GAAGTGCTGCACCCAAAAC	TCTGCCTGATGCTCTTGTTC
IL-8	GCTGCAGTTCTGGCAAGAGTA	ACCTTCTGCACCCACTTTTCC
MCP-1	TCTCCAGTCACCTGCTGCTAT	TGCTTCTTTAGGACACTTGCTG
PAI-1	TTGAGGAGAAGGGCATGG	CATCGGCCGTGCTG