Potential anti-fibrotic effects of MMP-2 and -9 inhibitor 1, Pepstatin A and Calpeptin in human precision-cut kidney slices

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Abstract

Introduction In an attempt to broaden the donation pool there has been a lot of interest in DCD kidney in recent years. DCD are usually inferior to DBD kidneys since they often undergo a serious warm ischemic injury. Previous research showed increased expression of matrix metalloproteinases 2 and 9, cathepsins D&L and calpains 1&2 during warm ischemic injury. These are all proteases involved in the microinflammatory response that drives IRI. Inhibition of these proteases could decrease the inflammatory and pro-fibrotic response, and ultimately increased graft survival of DCD kidneys. The aim of this study is to identify the anti-fibrotic effect of MMP2/9, Cathepsin D/L and Calpain1/2 inhibition using human precision-cut kidney slices.

Materials Human kidney tissue was gathered at the O.R. after a nephrectomy. Precision cut kidney slices were used to study the anti-fibrotic effects of MMP-2/-9 inhibitor 1, Pepstatin A (Cathepsin D&L inhibitor) and Calpeptin (Calpain 1&2 inhibitor) after 24 and 48 hours of incubation at 37°C. Viability was assessed with ATP analysis and collagen deposition was estimated with a Sirius red stain. Tissue damage was analysed with a PAS stain, based on glomerular structures, tubular dilatation and tubular necrosis.

Results All treated slices maintained viability during 48 hours of incubation, but did not show any significant difference with control after either 24 or 48 hours of incubation. Histological analysis with a PAS stain did not show a significant difference between treated and untreated slices in glomerular structures, tubular dilatation and acute tubular necrose. A Sirius red stain suggested in increase in collagen type 1 deposition in slices treated with MMP-2/-9 inhibitor 1, but showed contradictory results in slices treated with Pepstatin A and Calpeptin

Conclusion None of the inhibitory compounds appeared to have an anti-fibrotic effect on human precision cut tissue slices. More research needs to be done to explain deviations in results after MMP-2/-9-, Cathepsin D&L- and Calpain 1&2 inhibition.

Introduction

In recent years chronic kidney disease (CDK) has been recognized as a leading public health problem. CDK is currently prevalent in approximately 13% of the population worldwide, with numbers rapidly rising.[1,2] The rise is mainly driven by the increase of people with obesity, hypertension, diabetes mellitus and high age.[1] CKD has many different causes including ischemic injury, toxic insults or infections. Even genetic, endocrine or immunological diseases can play in import role in CKD.[3] If CKD progresses it often results in end stage renal disease (ERSD), ultimately leading to organ failure. Since treatment for ERSD is still limited, the number of patients in need of dialysis or transplantation keeps rising. Kidney transplantation is the most effective therapy for end-stage renal disease, but donor kidneys are scarce.[4] Kidneys used for transplantation are often kidneys donated after brain death (DBD) or circulatory death (DCD). DCD kidneys are inferior to DBD kidneys since DCD kidneys often endure a prolonged warm ischemia injury.[5,6,7] This injury causes delayed graft function (DGF) and primary non function (PNF), ultimately decreasing long term graft survival (LTGS).[7] Reducing the damage done by warm ischemic injury could increase LTGS, and provide a valuable additional source of donor organs.

During warm ischemic injury multiple processes and mechanisms are affected. There is for example an increased infiltration of inflammatory cells, such as macrophages and lymphocytes.[8,9] Together with injured tubular cells they release large amounts of inflammatory mediators, such as monocyte chemoattractant proteins (MCPs) and tumor necrosis factors (TNFs).[10] In turn these mediators signal additional inflammation, but also result in activation of relevant cell signaling pathways, such as the Notch and TGF-β -signaling pathways, that promote development of fibrosis.[10,11] Eventually, this will lead to the extracellular matrix (ECM) deposition that will induce the blocking of the renal interstitial capillaries and hypoxia. At this point a vicious circle is created that in time will lead to decreased kidney function, or even graft failure.[10]

Interfering in one of the activated signaling pathways could decrease the damage done by the ischemic injury, and increase LTGS of DCD kidneys. Earlier experiments revealed increased protease activity during WI in kidneys.[12,13] In this study three proteases are highlighted: Matrix metalloproteinases (MMPs), Cathepsins and Calpains. These are large families of proteases, but proteomics analysis showed increased activity mainly of MMP2 and 9, Cathepsin D and L and Calpain 1 and 2.

Matrix metalloproteinases 2 and 9 are part of a family of zinc-dependent proteases. They are found in almost all cell types, and are responsible for degradation and remodelling of collagens and other extracellular matrix proteins.[14] Several studies have demonstrated that MMP-2 and MMP-9 are upregulated after ischemia-reperfusion, and that MMP activation modulates renal microvascular permeability. [13,14,15] This is because these gelatinases have an especially high affinity to collagen type IV, an important element of basement membranes. Gelatinases are therefore essential for crossing through the endothelium by inflammatory cells.[14] This means that increased MMP expression during WI increases infiltration of inflammatory cells. Gelatinases also possess the ability to activate numerous pro-inflammatory agents, like interleukin 1B.[16] The activation of these proinflammatory

compounds together with the infiltration of inflammatory cells creates the essential condition for inflammation spreading, ultimately leading to more organ damage.

Proteomics analysis also showed increased cathepsin D and L activity during WI of the kidney. Cathepsin D and L are lysosomal proteases, play a critical role in the homeostasis of ECM deposition and. Their main physiological functions consist of degradation of intracellular and extracellular proteins. Cathepsin D is one of the major lysosomal aspartic proteases, and is essential for normal functioning of the autophagy-lysosomal system.[17] Fox et al., showed a reduction in fibrosis in two CKD mouse models after administration of Pepstatin A, a Cathepsin D and L inhibitor increased collagen degradation. They suggested a mechanism whereby Cathepsin D inhibition led to increased collagenolytic ability because of impaired lysosomal recycling.[18] So inhibition of Cathepsin D and L could cause increased extracellular activity of proteolytic enzymes, leading to more ECM degradation.

Beside MMPs and Cathepsins a third protease family showed increased activity after exposure of WI to the kidney: Calpains. Calpains are non-lysosomal cysteine proteases that are activated by calcium. During ischemia a calcium overload occurs in the cytosol and mitochondria, that would cause activation of calpains under normal conditions. However, during ischemia they are temporarily inhibited because of a low intracellular pH, caused by an increase in reactive oxygen species (ROS) After reperfusion, the increase in oxygen delivery and the pH normalization induce the activation of calpain. This leads, together with a large burst of ROS, to cell death through both apoptosis and necrosis.[19] Upon activation they are able to impair the energy production by cleaving structural and functional proteins of mitochondria.[20] They are also involved in structural remodeling and release of proapoptotic factors from mitochondria.[14]

Above mentioned mechanism explained that the up-regulation of matrix metalloproteinases 2 and 9, cathepsins D and L and calpains 1 and 2 are all involved in the microinflammatory response that drives IRI. Inhibition of these specific proteases could decrease the inflammatory and pro-fibrotic response, and ultimately increased graft survival.

Inhibitors for MMP2 and 9, Cathepsin D and L and Calpain 1 and 2 were selected based on previous research. MMP-2 and -9 inhibitor 1 will be used to inhibit MMP-2 and MMP-9.[20] Pepstatin A was selected as inhibitor for Cathepsin D and L , and Calpeptin will be used to inhibit Calpain 1 and 2.[17,21] Selected compounds will be tested in human kidney tissue.

However, obtaining healthy human kidney tissue is obviously challenging. This is why selected compounds will be tested in human kidney tissue obtained after a nephrectomy. During a nephrectomy all or a part of the kidney is removed, but only a small amount will be available for research. This means that there is need for an adequate in vitro model that can test the effect of the compounds, with only a limited amount of tissue. Over the years multiple methods have been developed to analyze compounds. Methods range from cell level, like organoids, to whole organisms. Organoids are multicellular structures derived from adult organs or pluripotent stem cells.[22] They can be used to analyze specific cells, but still lack large cellular diversity and tissue function. A unique method to test compounds are precision-cut tissue slices (PCTS). Thin slices are made with a mechanical slicer and incubated to maintain viability. During this process organ architecture is maintained, providing the possibility to study multicellular processes.[23] This method also requires a

very little amount of tissue. This is ideal for this experiment since only limited human tissue will be available.

The aim of this study is to identify the anti-fibrotic effect of MMP2/9, Cathepsin D/L and Calpain1/2 inhibition using human precision-cut kidney slices. This will be done by analyzing tissue viability, inhibitor activity and histological changes.

Materials and Methods

Experimental design pilot study

To determine the viability and general pipeline of human tissue slices a pilot study was conducted with two human kidney resections, obtained from nephrectomies. During the operation a call was made to us approximately 15 minutes before expected removal of the kidney from the body, which allowed for minimal WI time of the kidney tissue. At retrieval the resection was put on ice in University of Wisconsin cold storage solution (UW-CS, Belzers MPSå, Bridge to life Ltd., United Kingdom). Both kidneys underwent >60min of WI due to setbacks in the O.R. Precision-cut kidney slices were made and incubated for 24 and 48 hours at 37°C for the first kidney. This was extended to 96 hours for the second kidney. Medium was refreshed every 24 hours, and samples were taken at 24, 48, 72 and 96 hours. ATP analysis was performed to determine tissue viability.

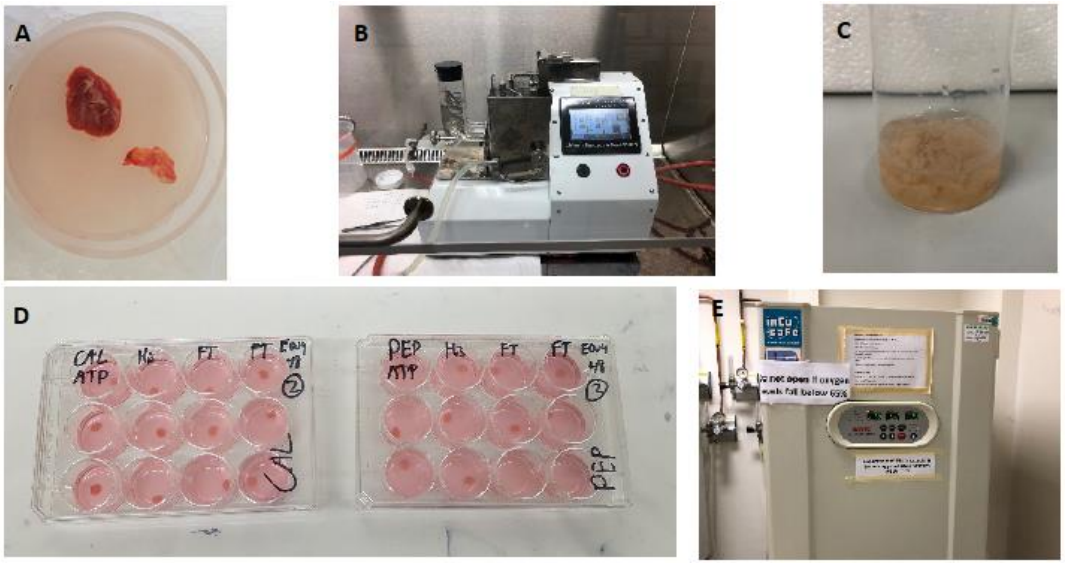

Figure 1 Experimental design: A. *Human kidney resection after retrieval at the OR.* ***B****. Krumdieck slicer* ***C.*** *Gathered slices stored in UW-CS.* ***D.*** *Plates filled on heated pads with medium, compounds and slices****. E.*** *Incubator set at 80% O_2/5%CO_2*

Experimental design experiment

The inhibitory compounds were added to the slices of the following kidneys when the slices from the pilot were determined positive. A total of four kidneys were studied to determine the anti-fibrotic effect of the selected compounds. Similar to the pilot study the tissue was gathered at the OR, and stored in UW-CS. Each kidney underwent a minimal WIT of 30 minutes. Precision-cut kidney slices were made and stored in UW-CS. Williams Medium E (1X) + GlutaMAX (Gibco) was used as medium. D-(+)-glucose (7.55mM) (Sigma-Aldrich, United Kingdom) was added to the medium to simulate *in vivo* blood sugar levels. Additionally 2mg/mL ciprofloxacin was added to prevent bacterial infections

during cell culture. Medium was incubated for 30 minutes (37°C/80%O_2/5%CO_2) to allow temperature and oxygen levels to rise. After 30 minute incubation compounds were added, this resulted in five different groups (Table 1). Slices were incubated for 24 and 48 hours at 37°C (80%O_2/5%CO_2). Medium was refreshed after 24h. After incubation samples were processed and analyzed for viability and histological changes

Groups	*n*	Mechanism
Control	4	Acts as a control
MMP-2/-9i	4	Small molecule that inhibits both Metalloproteinase 2 and 9.
Pepstatin A	4	Highly selective inhibitor of Cathepsin D and L.
Calpeptin	4	Inhibits both Calpain 1 and 2.
TGF-β	4	Acts as positive control by activating the TGF-β pathway.
MMP-2/-9i + TGF-β	1	Inhibition of Metalloproteinase 2 and 9 an activation of TGF-β pathway.
Pepstatin A + TGF-β	1	Inhibition of Cathepsin D and L an activation of TGF-β pathway.
Calpeptin + TGF-β	1	Inhibition of Calpain 1 and 2 an activation of TGF-β pathway.

Table 1. Groups that were tested during the experiment. *Control, MMP-2/-9i , Pepstatin, Calpeptin and TGF-β were tested in four kidneys. The last kidney was tested for three additional groups: MMP-2/-9i + TGF-β, Pepstatin + TGF-β and Calpeptin + TGF-β.*

Inhibitor compounds

Compounds were added at IC50 concentration that was provided by the manufacturer (Table 2). IC50 of MMP2 is 310nM, and IC50 of MMP9 is 240nM. A final concentration of 310nM was administered to ensure inhibition of both metalloproteinases. MMP-2/-9i nhibitor 1 was dissolved in DMSO, and diluted (0,03 µg/µL) Aliquots were made and stored at -20˚C to minimize repeated thawing. Pepstatin A was selected as inhibitor for Cathepsin D and L. Stock of pepstatin A was dissolved in DMSO, diluted (0,178 µg/µL), and aliquots were stored at -20°C. During preparation of plates 5uL was added to 1,3 mL medium, which resulted in a concentration of 1 µM (IC50) per slice.

Compound	**Stock concentration**	**Final concentration**	**Manufacturer**
MMP2/9 inhibitor	200 mg/mL	310 nM	Sigma Aldrich (CAS 193807-58-8)
Pepstatin A	1 mg/ml	1 µM	Sigma Aldrich (77170)
Calpeptin	5 mg/mL	52 nM	Sigma Aldrich (CAS 117591-20-5)
TGF-β	1 µg/mL	1.4 µM	Sigma Aldrich (T7039)

Table 2. Selected inhibitor compounds. *Both stock and final concentration are shown. Final concentration is added to 1,3 mL medium*

Calpeptin was selected as inhibitor for Calpain 1 and 2. Stock was diluted to 0.0049 µg/µL, aliquots were made and stored at -20˚C. 5 µL of diluted inhibitor in DMSO was added to the medium, with an end concentration of 52 nM (IC50) per slice. As a positive control TGF-β diluted in PSA/PBS (1 µg/mL) was used. An additional 5 µL of DMSO was added to get similar DMSO concentrations across all five groups.

Precision-cut kidney slices (PCKS)

Kidney resection was gathered at the O.R. and put in UW-CS solution. The resection was transferred into a larger tray with UW-CS, and excess fat was removed. A flat piece of cortex was removed, from which 6mm cores were drilled with a biopsy punch. Excess medulla was cut off. Precision-cut kidney slices were obtained using the Krumdieck tissue slicer (Alabama Research and Development, Munford, USA). The Krumdieck slicer was assembled and filled with Krebs-Henseleit buffer (KHB) for the slicing procedure. For three liters KHB preparation, 6,3 g NaHCO3 (B. Braun, Germany), 14,85 g D-(+)-glucose monohydrate (Merck, The Netherlands) and 7,14 g HEPES (Merck, The Netherlands) was

dissolved in 2,7 liters ultrapure water at 4°C. with 300 ml of 10x KHB diluted in ultrapure water to yield a final volume of three liters. The solution was kept on ice and oxygenated with carbogen for a minimum of 30 minutes. The pH was adjusted before use to 7,4 with 5M NaOH. During slicing the Krumdieck tissue slicer was cooled by recirculating 4°C water using a water bath. The weight of the slices was measured before each core, with a weight between 4-5 mg and an estimated thickness of 300 µm. Slices were quickly collected out of the KHB and put in back in 4°C UW-CS to maintain viability. Slices were then selected based on round and intact morphology and counted.

Incubation of PCKS

Slices were cultured in 1,3mL Williams Medium E (1X) + GlutaMAX (Gibco). 500mL was supplemented with 1,375g D-(+)-glucose (Sigma-Aldrich, United Kingdom) and 0,5mL ciprofluxine (2mg/mL). Medium was pre-incubated for 30 minutes at 37°C with 80% O_2 and 5% CO_2 to allow temperature and oxygen levels to rise. After 30 minute incubation compounds were in five different groups: Control, MMP-2/-9i , Pepstatin, Calpeptin and TGF-β. Plates were put on a heat matrass to prevent the medium from cooling down. The last kidney was tested for three additional groups: MMP-2/-9i +TGF-β, Pepstatin + TGF-β and Calpeptin + TGF-β. Slices were incubated for 24 (only ATP analysis) and 48 hours at 37°C (80% O_2/5%CO_2). Plates were refreshed after 24h.

At 24 and 48 hours three slices were individually stored in an Eppendorf with MiniBeads containing sonification solution (SONOP, consisting of 0.372 g EDTA in 130 mL H2O and NaOH (pH 10.9) + 370 mL 96% ethanol), and snap frozen in liquid nitrogen. At 48 hours three slices were put in a bracket and stored in 4% buffered formaldehyde for histological analysis. Another six slices were gathered for additional analyses, three slices were put in two Eppendorf cups and snap frozen in liquid nitrogen.

ATP determination

ATP concentration was determined with the ATP bioluminescence kit by Roche. Snap frozen samples in 1mL Sonop with Minibeads were homogenized with a minibead-beater for 4x45 seconds. Homogenized samples were centrifuged for 5 minutes (13 000 rpm) at 4°C , and supernatant was transferred to a new tube. During all steps the samples were kept on ice to prevent ATP breakdown. The left over sample was put in the stove at 37°C or 24 hours in order for excess water to evaporate. The remaining pellet was used for protein analysis. 5 µL of supernatant was added to a 96 wells plate in duplo, in combination with a positive control, blanco and calibration curve (Roche ATP assay kit). 45 µL Tris/EDTA buffer was added to each duplo and positive control. 50 µL luciferase was added and luminescence was measured using a luminometer (SynergyHT ATP protocol) at 0 and 5 minutes.

Protein determination

Protein concentration of each sample was determined with the Lowry protein assay (Biorad RC DC Protein Assay). Homogenized samples from ATP analyses one day 1 one were put in the stove at 37°C. After 24 hours 200 µL of 5M NaOH was added to each tube. Tubes were then incubated in a shaking water bath (37°C) for 30 minutes. After incubation 800 µL MQ water was added to reach a similar end concentration as with the ATP analyses. Samples were homogenized for 40 seconds with the minibead-beater, and put on ice. 5 µL of sample was added to a transparent 96 wells plate in duplo. After addition of protein reaction agents the plate was incubated in the dark for 15 minutes. Absorbance was measured at 650nm using the standard settings of Synergy's Lowry Assay protocol. Final ATP concentration was expressed as ATP/ug protein.

Histology

For each group 3 slices were placed into histology brackets after 48 hours of incubation and put in 4% formalin, where they were stored for a minimum of 24 hours. Tissue was then dehydrated through 70%, 80% and 95% alcohol, 45 minutes each. Followed by 3 changes of 100% alcohol 1 hour

each. Tissue was then cleared in xylene for 1 hour, and immersed in paraffin. After completion of the dehydration process the tissue was fixed in a paraffin block, and sliced into 4.5 µm paraffin sections using a microtome. Paraffin sections were put onto slides and put into the stove for approximately 7 hours at 37˚C to deparaffinize the slides.

First a periodic-acid Schiff (PAS) stain was performed. Slides were first rehydrated and stained with periodic acid solution (1.5 g in 250 mL demi water) for 5 minutes. After which slides were quickly rinsed in distilled water an stained in Schiff reagent for 15 minutes. Slides were then washed in running lukewarm tap water for 5 minutes, and counterstained in Mayer's hematoxylin. Another wash with running tap water was performed after which slides were dehydrated in three changes of 100% ethanol. Slides were then cleared in two changes of xylene and mounted with a coverslip using DePeX mounting medium (Serva, Germany)..

For the Sirius red stain the slides were first rehydrated. Slides were then stained for 1 hour in Picro SiriusRed solution, and washed in acetic acid solution (Abcam Picro-Sirius red stain kit, ab150681). Slides were dehydrated in two changes of 100% alcohol and mounted using DePeX mounting medium (Serva, Germany). Sirius red stained slides were scanned with a Hamamatsu for analysis of collagen deposition.

Periodic-acid Schiff stain analysis

Periodic-acid Schiff stained slides were analyzed with a microscope and quantified using a scoring protocol. Scores were given from 0 to 2 based on glomerular dilation, structural changes, tubular dilatation and tubular necrosis. Table 3 shows criteria for the scores given within the three categories.

Parameters	**Score**	**Qualification**
Glomerular dilatation and structural changes	0	- Glomeruli are still intact, clear clove-structure. - Capillaries have thin walls and are clearly visible.
	1	- Clove-structure is less present and glomeruli are not entirely intact. - Capillaries are less present and dilated, capillary walls are not clearly visible. - Heightened dilatation of the Bowmans space. Bowmans space can be filled with necrotic tissue.
	2	- Complete dilatation of the Bowmans space of 50-100% of the glomerulus' size. - Glomeruli have decreased in size and are crumpled up.
Tubular dilatation	0	- Tubules are not dilated. The brush border and tubule membranes are clearly visible
	1	- Tubules are 2 times their regular size. Intratubular space is visible and tubule membranes are no longer attached to each other. Tubular cells still have a regular size. The brush border is no longer visible.
	2	- Tubules have increased to >2 times their regular size. Tubular cells are often not visible and tubules themselves often have irregular shapes.
Acute tubular necrosis	0	- See score 0 tubular dilatation
	1	- Combined necrotic tissue with healthy tissue. Little thickening of the membranes, tubules contain some cells.

	2	-Vacuolization of cells, loss of brush border. Dilation of tubular lumen, mild interstitial inflammation. Epithelium of the proximal tubules is flattened and simplified. No clear tubular definition. Thickening of the tubule membrane. Tubules contain many cells.

Table 3, Protocol for scoring slides stained with periodic-acid Schiff reagent. Scores are given to three different categories: Glomerular structures, tubular dilatation and tubular necrosis. Criteria are shown for each score.

Sirius Red stain analysis

The Sirius red stain was quantified with a positive pixel analysis in Image scope. For each slide four random high-quality pictures were made and each picture was analyzed individually. A custom set of parameters was used with hue with and value as 0 for analysis of the color red. A color saturation threshold of 0.2 was used to eliminate the background, and ranges were set to quantify different pixels: weak positive pixels (0-131nm), positive pixels (131-155nm) and strong positive pixels (155-189nm). Strong positive pixels as percentage of total amount of pixels was used as an indication of collagen deposition (%ppa).

MMP activity assay

MMP activity was measured with a gelatinase assay kit (Bio-Rad Gelatinase zymography assay kit). Snap frozen samples (5-10mg) were homogenized with 100 µL cell lysis buffer and incubated on ice. Supernatant was transferred to precooled Eppendorf's and stored on ice. Lysate was added to a 96 wells plate with buffer and substrate in duplo. Concentration of added lysate was determined with a pilot study. Fluorescence was measured at Ex/Em 490/520nm in kinetic mode at 37C for 1-2 hours. Amount of protein in the lysate was measured with the BCA protein assay kit. Delta RFU was obtained with a FITC standard curve. Sample gelatinase activity was calculated and corrected for protein concentration. Final sample gelatinase activity was expressed as U/mg protein, where 1 U is the amount of Gelatinase that is required to cleave the gelatinase substrate and release 1 pmol of Fluorescence per minute.

Statistics

Statistical analysis was performed with an unpaired two-sample t-test in GraphPad prism. T-tests were performed with no assumed similar standard deviations when comparing control to treated. Results are expressed as mean plus standard deviation. A difference was considered significant when $P<0.05$

Results

Results pilot study

ATP analysis was performed to determine the viability of the human precision-cut kidney slices (Figure 2). ATP per protein showed a significant increase after 24 hours of incubation in slices of both kidneys (P=0.0016). After 48 hours of incubation one of the kidneys appears to show an increase in ATP concentration, whereas the second kidney shows a decrease. ATP concentration of both kidneys was still significantly increased compared to the 0h samples (P=0.048). Precision-cut kidney slices of one kidney were incubated for additional 24 or 48 hours, but did not show a significant difference compared to samples taken before incubation.

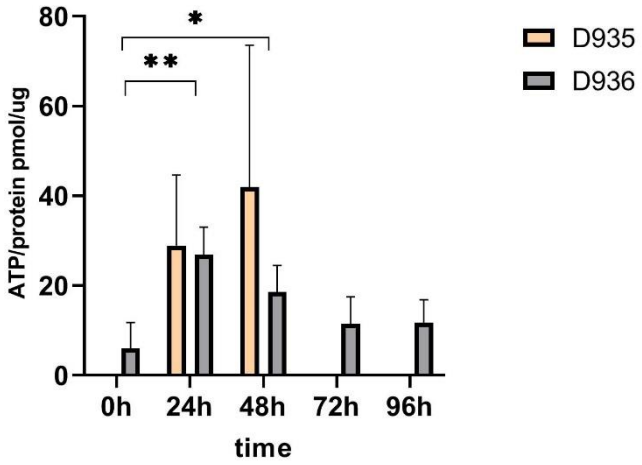

Figure 2, *ATP concentration measured in ATP per protein (pmol/µg) in human precision-cut kidney slices, pilot study (n=2). First kidney had no samples for timepoint 0h. The second kidney was measured for an additional 24 and 48 hours.*

Results protease inhibitors

Metalloproteinase 2/9 inhibitor 1

ATP Figure 3A shows the ATP per protein of the four kidneys treated with MMP2/9 inhibitor 1. ATP per protein is measured in pmol/ug. ATP levels at time point 0h were significantly lower for all four kidneys. After 24-hour incubation ATP per protein levels rose significantly, both for the control and MMP treated group (P=<0.0001). Kidney one and four showed a decrease in ATP/protein after 48h incubation. Kidney 3 showed a slight increase after 48h. Only kidney two showed a significant difference in ATP concentration between the control and treated group after 48h incubation (P=0.014). Figure 3B shows the results of all kidneys together. No significant differences were observed between the control and treated group after both 24 or 48 hours of incubation.

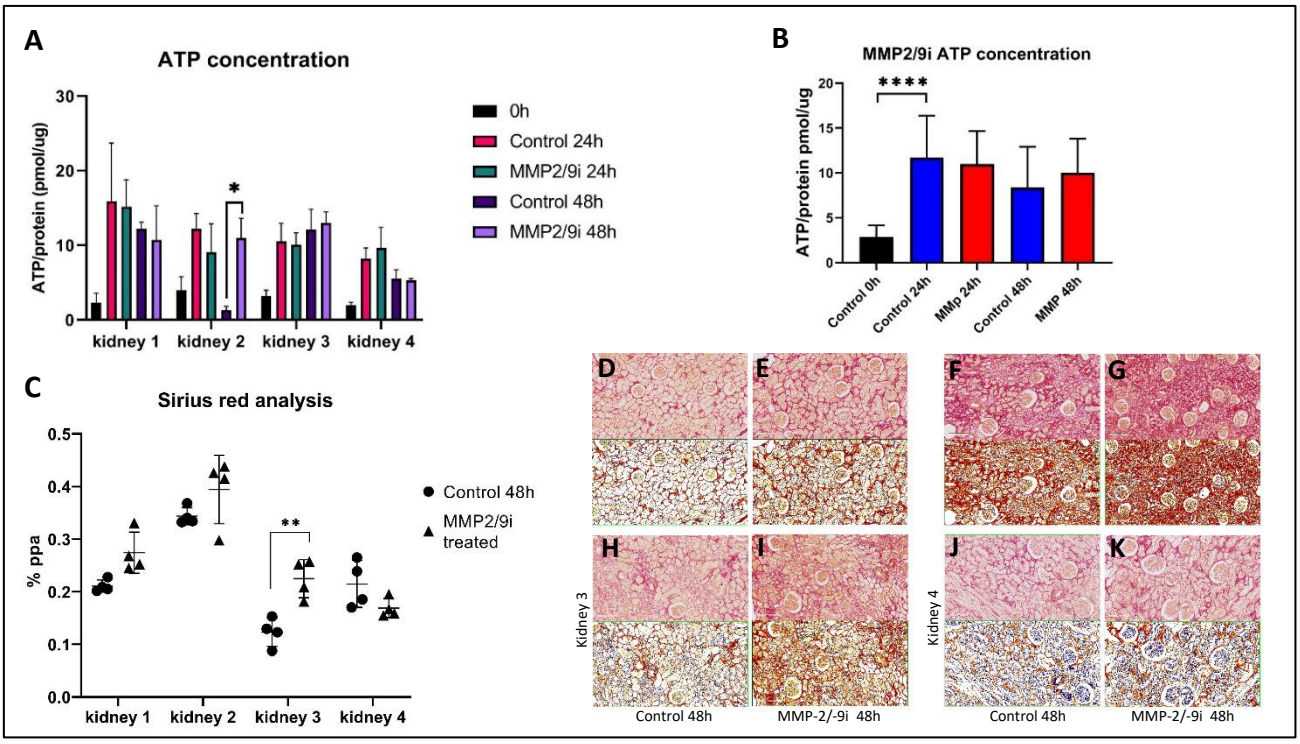

Figure 3, A. *ATP analysis of PCKS treated with MMP-2/-9i 1 (n=4). Results are show for each individual kidney. ATP concentration is measured in pmol/µg.* ***B.*** *ATP analysis of PCKS treated with MMP-2/-9i 1 (n=4). Black rods indicate no incubation. Blue indicate no treatment, red indicates treatment with MMP-2/-9i 1* ***C.*** *Quantification of Sirius red expressed as % positive pixels (n-4).* ***D-K.*** *Screenshots of histological slides with Sirius red stain. Lower half shows the positive pixel analysis (n=4).*

Sirius red analysis Precision cut kidney slices treated with MMP2/9 inhibitor of kidney three showed a significant increase in %ppa after 48 hours of incubation (P=0.016)(Figure 3C,I). MMP-2/-9i treated slices of kidney two also appear to have and increased %ppa, however this difference was not significant.

PAS analysis Slides stained with PAS were quantified with a scoring protocol shown in Table 3. Slices treated with MMP2/9 inhibitor showed no significant differences in both tubular dilatation and glomerular structure (Figure 4C,D). Control slices incubated for 48 hours showed a significant

increase in tubular necrose compared to slices at timepoint 0h. (P=0.0012) Slices treated with MMP-2/-9i appear to show a decrease in tubular necrosis after 48 hours (Figure 4E).

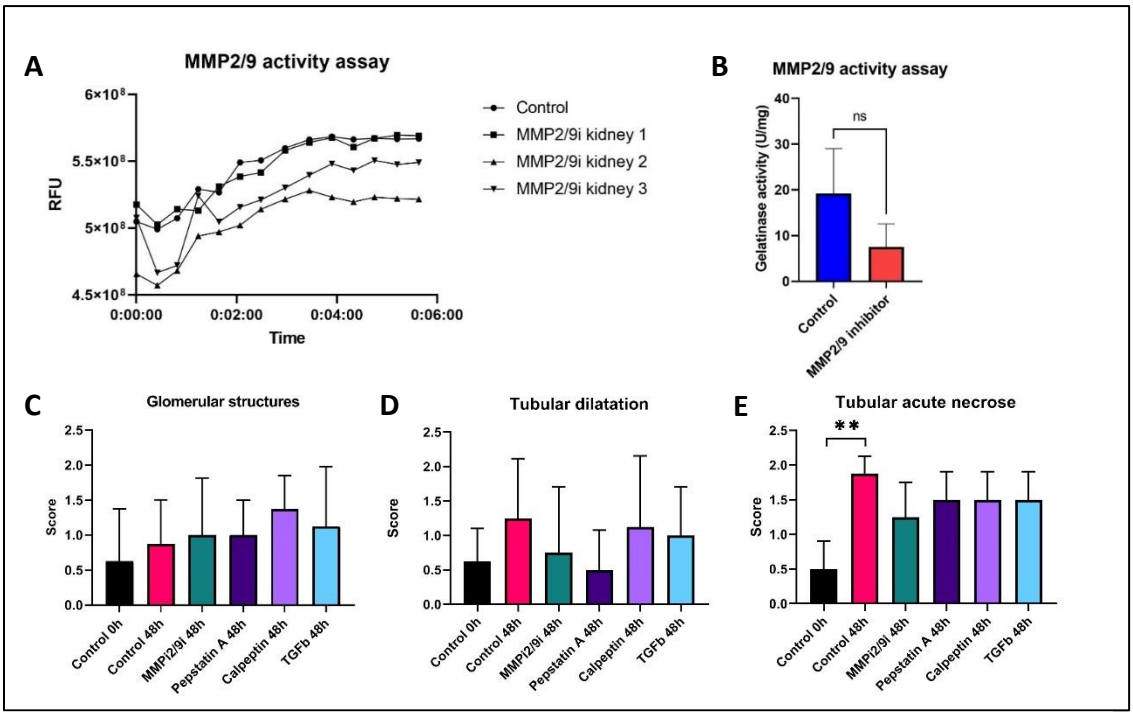

Figure 4, A. *MMP activity assay of MMP-2/-9i treated PCKS, shown as RFU per minute for each individual kidney (n=3)* ***B.*** *MMP activity assay measured as gelatinase activity in U/mg protein, where 1 U is the amount of gelatinase that is required to cleave a gelatinase substrate and release 1 pmol of Fluorescence per minute.* ***C.*** *Scores were given to PAS stained slices based on tubular dilatation,* ***D;*** *tubular necrosis and* ***E;*** *glomerular structures (Table 2).*

Inhibitor activity assay To test for inhibitor activity a gelatinase protein assay was performed. Figure 4A shows the activity of MMP2/9 measured in FTU, and gelatinase activity is shown in figure 4B. Although both figures appear to show a decreased MMP activity in the MMP-2/-9i treated group, there was not a significant difference compared to the control group.

Pepstatin A

ATP Figure 5A shows the results of ATP per protein of PCKS treated with the cathepsin inhibitor Pepstatin A. Similar to the group treated with MMP2/9 inhibitor 1, ATP/protein increases significantly from zero hour to 24 hours (P=<0.0001). At 48h incubation most slices showed a slight decrease in ATP per protein. A significant difference is observed between the control and Pepstatin A treated slices of kidney 2 after 48 hours incubation (P=0.042). However, when all kidneys are grouped together no significant differences are shown between control and treated after 48 hour incubation (Figure 5B).

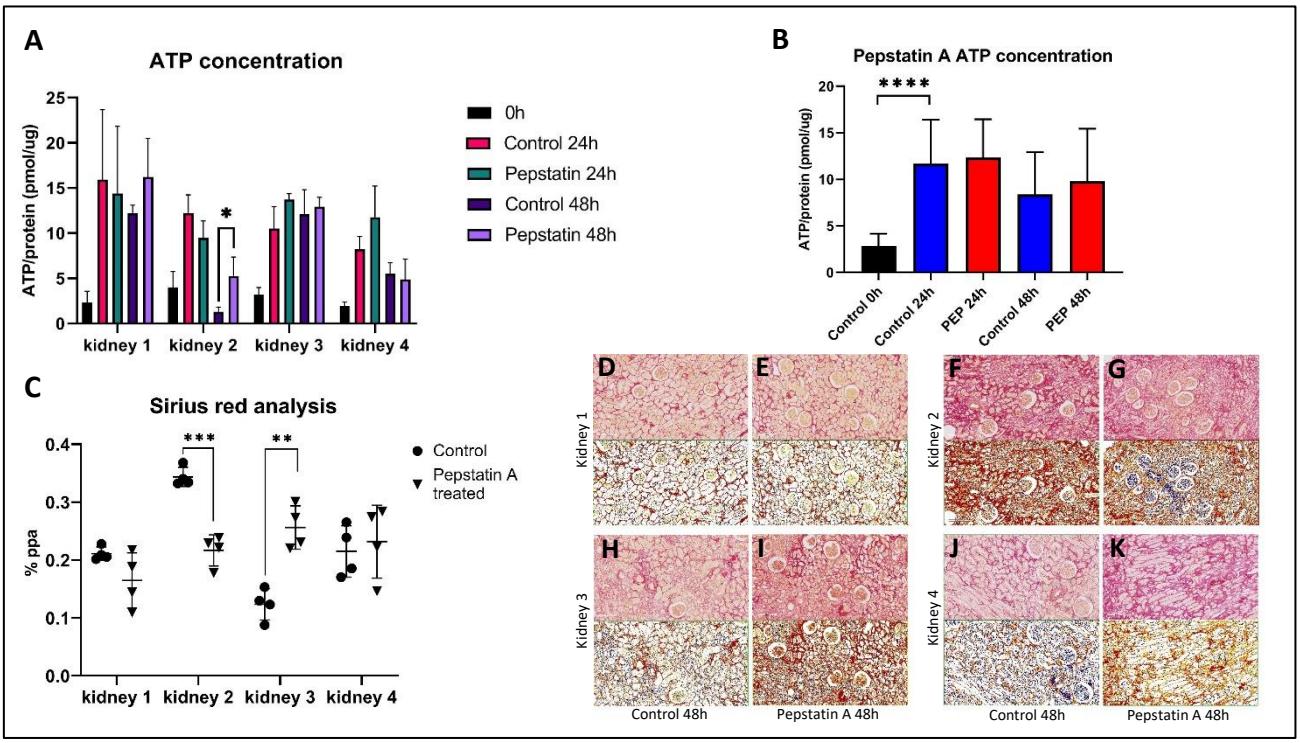

Figure 5, A. *ATP analysis of PCKS treated with Pepstatin A (n=4). Results are show for each individual kidney. ATP concentration is measured in pmol/µg.* ***B.*** *ATP analysis of PCKS treated with Pepstatin A (n=4). Black rods indicate no incubation, blue indicate no treatment and red indicates treatment with MMP-2/-9i 1* ***C.*** *Quantification of Sirius red expressed as % positive pixels (n-4).* ***D-K.*** *Screenshots of histological slides with Sirius red stain. Lower half shows the positive pixel analysis (n=4).*

Sirius red analysis Kidney two showed a significant decrease in percentage positive pixels in the Pepstatin A treated slices (P=<0.0001) (Figure 5C,G). Interestingly, kidney three showed a significant increase in %ppa in the treated group (P=0.004) (Figure 5C,I). Kidney one and four did not show a significant difference between the control and treated slices.

PAS analysis Slices treated with Pepstatin A showed no significant differences in either glomerular structure, tubular dilatation or glomerular structure after 48 hours of incubation. (Figure 4C-E)

Calpeptin

ATP Figure 6A shows the results of ATP per protein (pmol/ug) of PCKS treated with calpeptin. After 24h incubation the slices showed a significant increase in ATP concentration for all kidneys (P=<0.0001). However, no differences were observed between control and treated. After 48 hour incubation the slices showed a slight decrease in ATP per protein compared to 24h incubation, but no significant differences were observed between control and treated. When all kidneys were grouped together no significant differences are shown between control and treated after 48 hour incubation (Figure 6C)

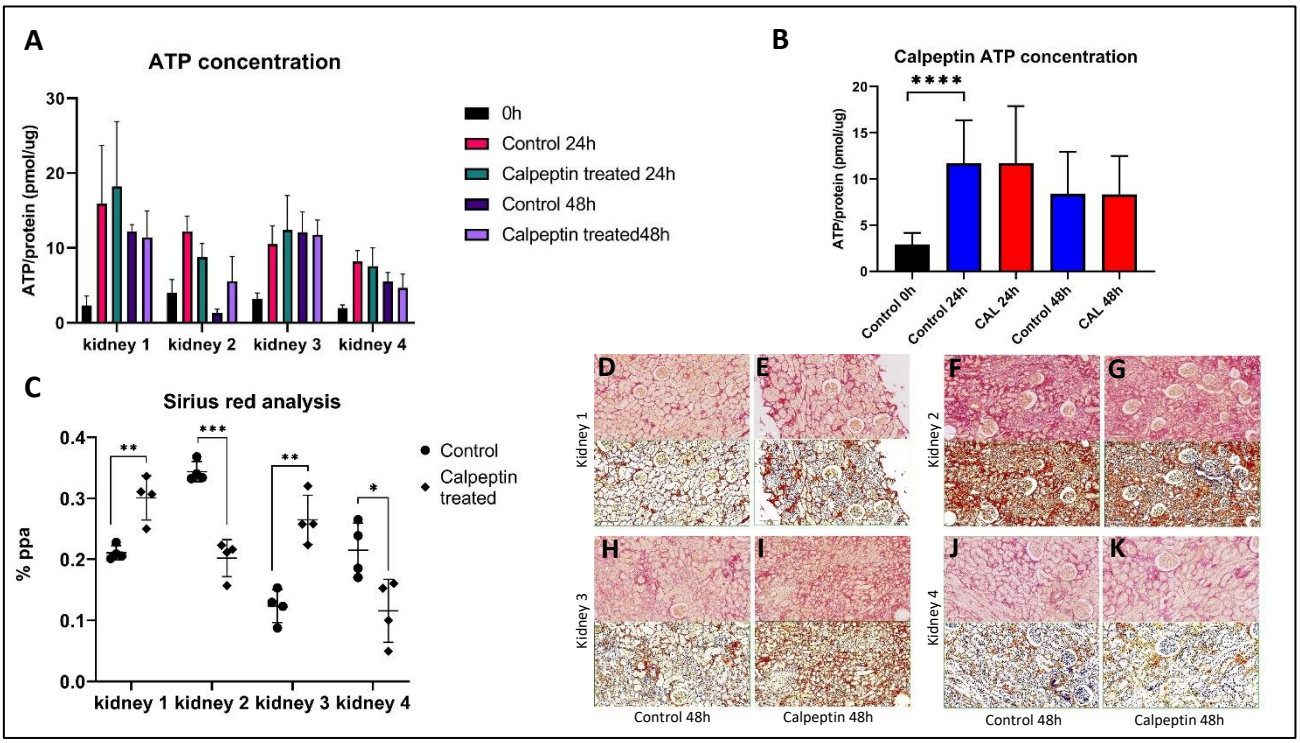

Figure 6, A. *ATP analysis of PCKS treated with Calpeptin (n=4). Results are show for each individual kidney. ATP concentration is measured in pmol/µg.* ***B.*** *ATP analysis of PCKS treated with Calpeptin (n=4). Black rods indicate no incubation. Blue indicate no treatment, red indicates treatment with Calpeptin* ***C.*** *Quantification of Sirius red expressed as % positive pixels (n-4).* ***D-K.*** *Screenshots of histological slides with Sirius red stain. Lower half shows the positive pixel analysis (n=4).*

Sirius red analysis Both kidney one (P=0.006) and three (P=0.0007) showed a significant increase in %ppa in slices treated with calpeptin (Figure 6C,E,I). However, kidney two (P=0.003) and four (P=0.02) showed a significant decrease in %ppa in treated slices (Figure 6C,G,K).

PAS analysis Slices treated with Calpeptin showed no significant differences in either glomerular structure, tubular dilatation or glomerular structure after 48 hours incubation (Figure 4C-E).

TGF-β

ATP Figure 7A shows the results of ATP per protein (pmol/µg) of PCKS treated with Calpeptin. Each kidney showed a significant increase in ATP concentration after 24h of incubation (P=<0.0001). Kidney two showed a significant decrease in ATP per protein in slices treated with TGF-β after 24 hour incubation (P=0.048). Interestingly, after 48 hours of incubation kidney 2 showed a significant increase in ATP per protein in TGF-β treated slices (P=0.025). When all kidneys are grouped together no significant differences were observed between control and treated after 48 hour incubation (Figure 7B).

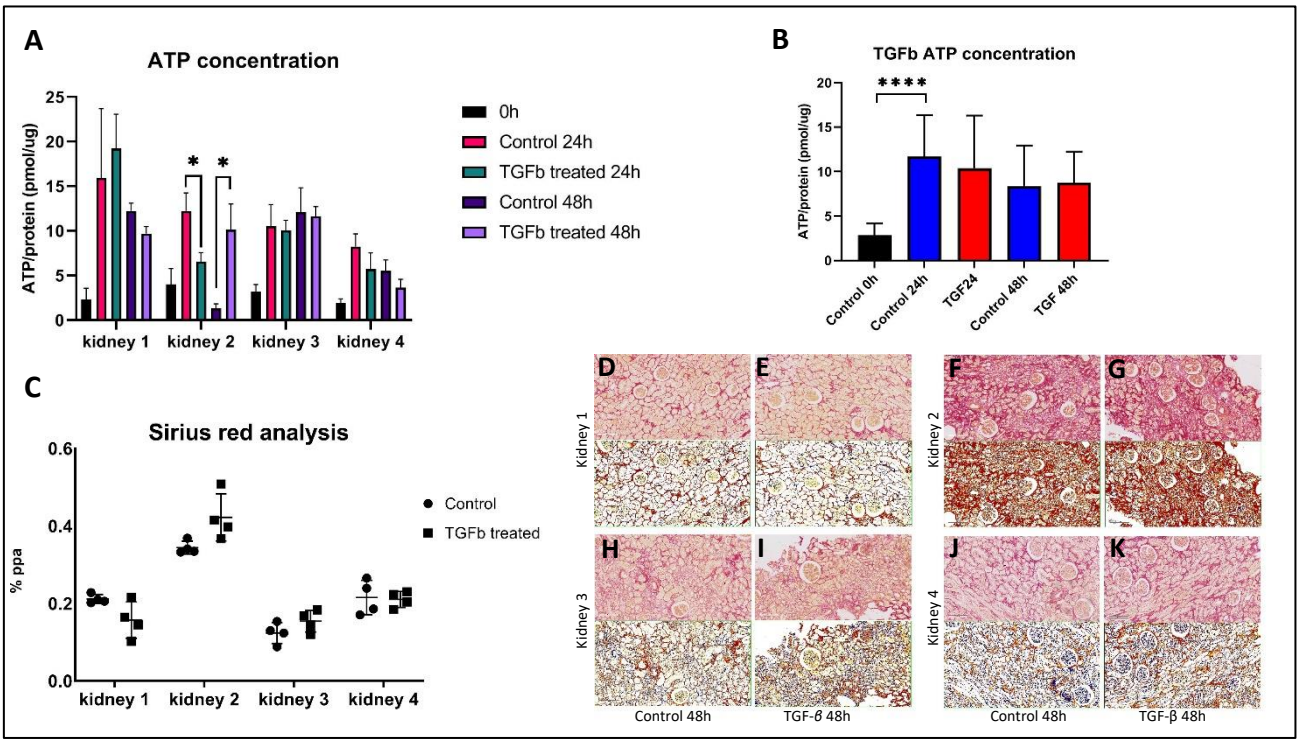

Figure 7, A. *ATP analysis of PCKS treated with TGF-β (n=4). Results are show for each individual kidney. ATP concentration is measured in pmol/µg.* ***B.*** *ATP analysis of PCKS treated with TGF-β (n=4). Black rods indicate no incubation. Blue indicate no treatment, red indicates treatment with TGF-β* ***C.*** *Quantification of Sirius red expressed as % positive pixels (n-4).* ***D-K.*** *Screenshots of histological slides with Sirius red stain. Lower half shows the positive pixel analysis (n=4).*

Sirius red analysis TGF-β was added as a positive control, but did not show significant differences in %ppa between control and treated. Kidney two and three appeared to show a slight increase in %ppa, although both differences were non-significant.

PAS analysis Slices treated with TGF-β showed no significant differences in either glomerular structure, tubular dilatation or glomerular structure after 48 hours of incubation (Figure 4C-E).

<u>MMP-2/-9i , Pepstatin A and Calpeptin in addition to TGF-β</u>

ATP An additional experiment was performed where TGF-β was added in addition to the inhibitors. Similar to the other experiments the ATP concentration was significantly increased for the control group after 48 hours (P=<0.009) (Figure 8A). Both Pepstatin A + TGF-β and Calpeptin + TGF-β did not show significant differences with the group treated with TGF-β. Additionally, the ATP concentration appeared to be lower than the control group after 48 hours incubation. MMP-2/-9 inhibitor + TGF-β showed a significantly increased ATP concentration compared to slices treated with TGF-β slices (P=0.009), but not with the control group.

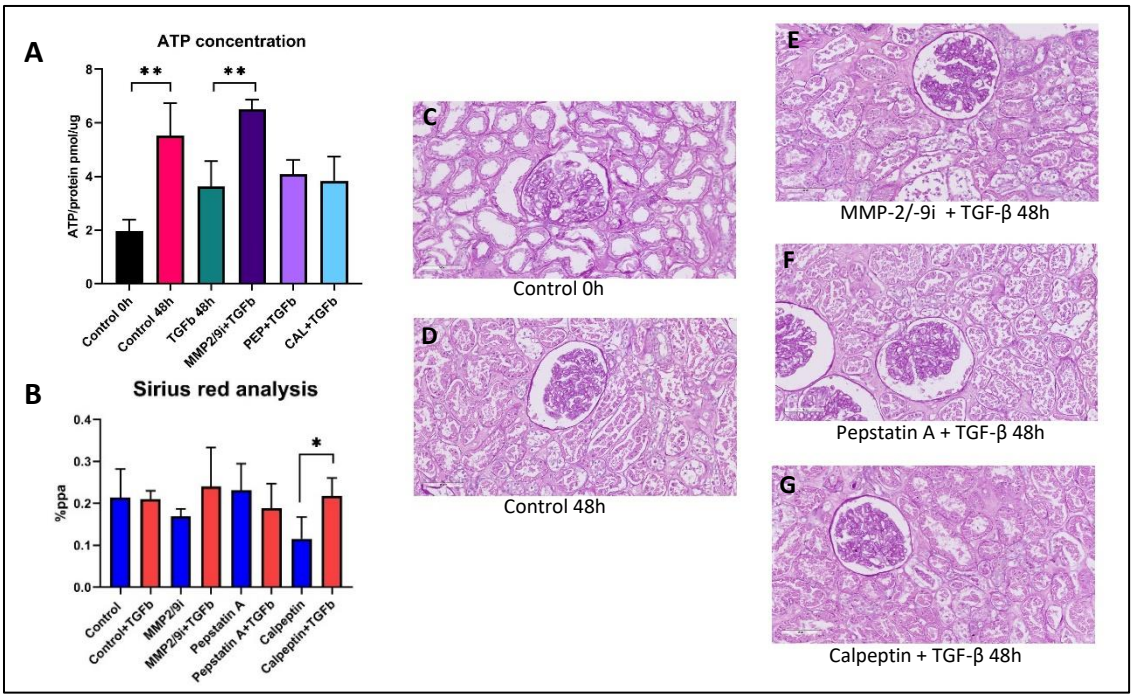

Figure 8, A. *ATP analysis of PCKS treated with either MMP-2/-9i , Pepstatin A or Calpeptin, in addition with TGF-β (n=1). PCKS treated with only TGF-β are also shown as an additional control. ATP concentration is measured in pmol/µg.* ***B.*** *Quantification of Sirius red expressed as % positive pixels (n=1)* ***C.*** *PAS stain of control group that were samples at time point 0h,* ***(D)*** *and after 48 hours of incubation.* ***E.*** *PAS stain of slices treated with MMP-2/-9i + TGF-β,* ***(F)*** *Pepstatin A + TGF-β or* ***(G)*** *Calpeptin + TGF-β after 48 hours of incubation.*

Sirius red analysis Figure 8B shows the Sirius red analysis of slices treated with TGF-β and either MMP-2/-9i , Pepstatin A or Calpeptin (n=1). Slices treated with Calpeptin + TGF-β showed significantly increased %ppa compared to slices treated with Calpeptin (P=0.0231). No significant differences were observed between slices treated with MMP-2/-9i or Pepstatin A and slices with both TGF-β and MMP-2/-9i or PepstatinA respectivly.

PAS analysis Slices treated with both inhibitor and TGF-β are not shown in Figure 4C-E since only one kidney was tested. Figure 8C shows PAS stained slices at timepoint 0h. Glomulurar structers were completely intact, and there was no acute tubular necrosis. However, there was a slight increase in tubular dilatation. After 48 hour incubation control slices showed acute necrosis and increased dilatation in almost all tubules (Figure 8D). Glomeruli were decreased in size and crumpled. Capillaries were less present and dilated. Slices treated with TGF-β and MMP-2/-9i , Pepstatin A or Calpeptin showed similar tubular dilatation to untreated slices. MMP-2/-9i + TGF-β and Calpeptin + TGF-β treated slices showed slightly less tubular necrosis compared to control after 48 hour incubation. Glomerular structures were equally damaged in both treated and control slices.

Discussion

The aim of this study was to identify the anti-fibrotic effect of protease inhibition using human precision-cut kidney slices. The following three compounds were studied: MMP2/9 inhibitor 1, Peptstatin A and Calpeptin. MMP2/9 inhibitor 1 had shown positive effects when added either before or after IRI, and was therefore used to inhibit Metalloproteinase 2 and 9.[20] Pepstatin A was used to inhibit Cathepsin D and L, because it had shown promising results in reduction of IRI in previous research.[21] Lastly, Calpeptin was used to inhibit both calpain 1 and 2.[17]

Human kidney tissue was obtained at the O.R. after a nephrectomy. Only a small amount of tissue could be gathered, which is why precision cut kidney slices were used as an ex vivo model to study the compounds. The PCKS model allows for use of only a very small amount of tissue, which was ideal for this study. Since there was little knowledge about the use of human tissue for precision cut kidney slices a pilot was performed to test viability of slices after 24 and 48 hours of incubation. ATP concentrations after 24 and 48 hours were significantly increased compared to samples taken before incubation, suggesting that the slices regained some metabolic function. Incubation was continued for 72 and 96 hours for one of the kidneys, but did not show a significant difference compared to 0h samples. ATP concentrations of the human slices at 24 and 48 hours were comparable with slices of porcine tissue in previous research, and were therefore considered viable for 48 hour incubation.[24]

ATP

After the pilot study inhibitors were selected based on previous research.[17,20,21] They were dissolved in DMSO and added at IC50 concentration. Similar to the pilot ATP concentration was used to determine the viability of the PCKS. Human PCKS slices were incubated for 24 and 48 hours. Control PCKS appeared to establish ATP concentrations during incubation, and remained viable for 24 and 48 hours when incubated. These findings were in line with previous research that studied viability of slices after incubation. Luangmonkong et al. showed in 2017 that viability of rat and human precision-cut renal slices could be maintained for up to 72 hours, and Stribos et al., observed optimal incubation period of 48 hours in murine PCKS.[16,25]

Results from the control slices were then compared with treated slices to detect for toxicity of the compounds. ATP per protein results showed that treated slices with either MMP-2/-9i , Pepstatin A or Calpeptin were still viable after 48 hour incubation (Figure 3B, 5B, 6B and 7B) This meant that the added inhibitor concentrations were not toxic, but the fact that treated slices were not significantly different compared to control also meant that the inhibitors do not provide an increase in viability.

This was expected for the slices treated with TGF-β (Figure 7B), since Poosti et all previously showed no significant effects of TGF-β on tissue viability in human PCKS.[26] MMP-2/-9 inhibition showed no significant differences after 48 hour incubation compared to control (Figure 3B). Previous research showed increased MMP-2/-9 expression during WI increases infiltration of inflammatory cells.[13] So inhibition of MMP-2 and -9 should have decreased the inflammation. This in turn was expected to increase tissue viabilty, but this was not observed in the measered ATP concentration. It is unclear as to why this happened.

As mentioned before, Cathepsin D&L inhibition by Pepstatin A also showed no significant differences compared to control, while previous research showed reduced ATP content after Cathepsin L inhibition.[27] This could be explained by the fact that recent research showed that

Cathpesin D and L inhibition mainly decreases IRI by reducing macrophage and neutrophil infiltration, and by decreasing pro-inflammatory cytokine production.[28] These result would not be directly visible in ATP concentration changes. Additionally, Cathepsin D and L inhibition was not measured, which could mean that the added inhibitor concentration was too low.

Histology

The Sirius red stain showed contradictory results; Inhibition with calpeptin suggested an increase in collagen deposition in two of the kidneys, but a suggested a decrease in the other two kidneys (Figure 6C). This means that additional research will have to be done to get conclusive results of calpeptin on collagen deposition. Similarly Pepstatin A treated slices suggested increased collagen deposition in one kidney, and a decreased collagen deposition in the other (Figure 7C). This was unlike previous research, which showed a reduction in fibrosis because of increased collagen degradation.[21] However, this research was performed in murine tissue, and Pepstatin A was administered days before resection of the kidney. This might explain the differences that were observed.

Strangely, the only group that did not show any significant differences was the TGF-β group. This group was added as appositive control for fibrogenesis, and collagen type 1 deposition. Previous research in human PCKS showed a significant increase of α-SMA, fibronectin and collagen I in slices treated with TGF-β in a similar human PCKS model.[26] It is unclear why different results were observed in out study. An possible explanation could be that they used healthy kidney tissue, whereas the tissue used in this study was already very fibrotic.

The PAS stain did not show any significant differences in glomerular structures, tubular dilation and acute tubular necrosis between treated and untreated PCKS (Figure 4C-E). Indicating that inhibition with MMP-2/-9 inhibitor, Pepstatin A or Calpeptin did not influence the tissue damage that occurs during incubation for 48 hours. However, this might be because incubation for 48 hours was simply too long. Even though ATP concentrations proved to be viable, control slices showed acute necrosis in almost all tubules, which in turn were all dilatated. Furthermore, glomeruli were crumpled up and almost no capillaries were visible. These findings indicate that the damage to the PCKS might be too much, and that 48 hour incubation might be too long. Additional histological research after 24 hours of incubation could provide interesting results, since less damage might have been afflicted.

Gelatinase assay

To test the effectiveness of MMP-2/-9i a gelatinase activity assay was performed (Figure 4A,B). The assay was performed for only three of four kidneys, since the last kidney was obtained last minute. A small amount of sample was added to a gelatinase buffer, and a reconstituted gelatinase substrate was added shortly before measuring fluorescence. An enzyme positive control was added to test whether cleavage of the gelatinase substrate would produce fluorescence. A pilot was performed with different concentrations to ensure that readings of the tested samples are within the standard curve range. In the pilot we also tested different concentrations of the positive control, since the protocol did not provide a clear value of positive control that should be used (1-10µL/well).

Interestingly only a concentration of 1µL/well positive control gave expected results based on the provided protocol. Higher concentrations of positive control showed a decrease in fluorescence measured over time, indicating that the kit might not be working correctly.

According to the protocol the samples should be measured for 2 hours, and should show a slowly increasing production of fluorescence. However, In the tested samples there is increase of

fluorescence for only 5 minutes in both control and MMP-2/-9i treated samples. This could mean that there is too much gelatinase activity, and that all substrate is cleaved within these 5 minutes. So a lower concentration of sample was used to decrease the number of enzyme, which should increase the time needed to cleave all the substrate. However, this did not occur. When these lower concentrations were measured there was little to no difference with a negative control (no substrate), indicating that there was no gelatinase activity.

So a high concentration showed activity for 5 minutes, but a lower concentration showed little to no activity for both control and MMP-2/-9i treated slices (Figure 4A,B). An explanation could be that the substrate of the kit was not working correctly. Since the positive control was not working correctly either, this is most likely the case.

However, even though the kit might not have worked properly, results still indicated that there was indeed inhibition of MMP-2 and -9. Two out of three kidneys showed a very large decrease in gelatinase activity, but kidney three leveled out a significant difference because of a small sample size (n-3).

Recommendations for further research

Results from the different analyses were difficult to interpret since they were often contradictory, mostly likely because of the high heterogeneity of the tissue. All received resections were very different in color, size and of course condition, and were not allowed to know any additional information about the patients. It is highly likely that the resected kidney is usually in stage 5 of CDK, or carries malignant tumor cells. This means that the kidney lost pretty much all of its function, and is definitely not in optimal state. Both cellular and extracellular matrix homeostasis and functioning are most likely affected, questioning whether the kidneys give a good representation of the effect of the inhibitors. To test this a future study could consider to test different concentrations of the inhibitors in a porcine precision cut kidney slices model. This would allow us to observe the effect of the inhibitors in healthy tissue, and also gives the opportunity to establish a most optimal concentration. This would be tedious to establish in the human model, since the tissue that is obtained is very limited. The question then of course becomes whether the results shown in porcine tissue would be translatable to human tissue. But since the human tissue is very heterogeneous, it might be favorable to observe the anti-fibrotic effects of the inhibitors in a more controlled environment.

Not all three inhibitors were tested for effectiveness. Only MMP2/9 activity was tested with an gelatinase activity assay, and appeared to show a decreased MMP2/9 activity in the slices treated with MMP-2/-9i . Both Pep statin A and Calpeptin were not analyzed for activity. This means that the added concentration of inhibitors might not have been optimal, and future research will have to be done to prove this.

A Sirius red stain was performed to estimate collagen deposition of collagen type 1 in the PCKS. However, In most studies an additional western blot is performed to test for collagen type 1, since the stain alone does not always provide accurate results. So an additional western blot, or a human Pro-Collagen I alpha ELISA, should be performed in the future to get a better estimation of collagen deposition in the PCKS.

Lastly, a bigger sample size is could provide insights to some of the questions still at harm. For example why Pepstatin A showed a significant decrease in estimated collagen deposition two kidneys, but a significant increase in the other two kidneys. However, because the tissue obtained from a nephrectomy is very heterogenous similar results might be observer. This means that studies

in healthy human kidney tissue are needed to observe the anti-fibrotic effects of MMP-2/-9 inhibitor 1, Pepstatin A and Calpeptin.

Conclusion

The aim was to study the anti-fibrotic effects of three different protease inhibitors, MMP-2/-9 inhibitor 1, Pepstatin A and Calpeptin on human precision cut kidney slices. The effect was observed by looking at ATP concentrations, inhibitor activity and morphology. All treated slices maintained viability during 48 hours of incubation, but did not show any significant difference with control after either 24 or 48 hours of incubation. Histological analysis with a PAS stain did not show a significant difference between treated and untreated slices in glomerular structures, tubular dilatation and acute tubular necrose. A Sirius red stain suggested in increase in collagen type 1 deposition in slices treated with MMP-2/-9 inhibitor 1, but showed contradictory results in slices treated with Pepstatin A and Calpeptin. Additionally, slices treated with TGF-β showed no significant difference when compared to untreated slices, indicating that the analysis method might not be optimal. Future research will have to be performed to show the effect of the inhibitory compounds on collagen type 1 deposition. None of the inhibitory compounds appeared to have a anti-fibrotic effect on human precision cut tissue slices.

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