TITLE: Mass red blood cell extravasation and tubular uptake results in toxic injury to the tubules during kidney ischemia from venous clamping

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ABSTRACT (250)

Vascular congestion represents densely packed red blood cells (RBC) in the kidney circulation. In this study we tested the hypothesis that ‘vascular congestion directly promotes tubular injury’.

All studies were performed in male and female Wistar-Kyoto rats. Vascular congestion and tubular injury were histologically examined between renal venous clamping, renal arterial clamping and venous clamping of blood free kidneys. Vessels were occluded for either 15 or 45 minutes without reperfusion.

We found that venous clamping resulted in greater vascular congestion than arterial clamping, particularly in the outer-medullary region ($P<0.001$). Venous clamping resulted in significant tubular injury, including cell swelling, tubular necrosis and luminal cast formation following 45-minutes of occlusion. Tubular injury was significantly less following 45 minutes of arterial clamping ($P<0.001$). Numerous red droplets were observed within tubular cells. These were most prominent in injured cells and following venous clamping. Electron microscopy identified these droplets as RBCs and indicated that RBCs from congested renal capillaries were extravasated and taken up by tubular cells. Labelling RBCs with a cell permanent dye confirmed tubular uptake of RBCs. Demonstrating that tubular injury was due to RBC uptake, tubular injury was markedly reduced in blood free kidneys following venous clamping ($P<0.001$).

Our data demonstrate that congestion of the kidney results in the rapid, mass extravasation and uptake of RBCs by tubular cells causing toxic injury to the tubules. Tubular toxicity from extravasation of RBCs appears to be a major component of tubular injury in ischemic AKI which has not previously been recognized.

INTRODUCTION

Vascular congestion or red cell trapping represents the formation of densely packed red blood cells (RBC) in the renal circulation[1, 2]. Vascular congestion of the outer-medullary region is a hallmark of ischemic acute kidney injury (AKI) with acute tubular necrosis (ATN) in humans at autopsy[3-8] and is also present in animal models following ischemia reperfusion (IR) from arterial clamping, including in pigs, dogs, rats and mice[9-12]. Vascular congestion of the renal cortical circulation also occurs during periods of ischemia however, this rapidly resolves following restoration of kidney perfusion[1, 13].

Our laboratory has recently reported that vascular congestion is associated with severe tubular injury in the renal outer-medullary region occurring and that this occurs within two hours of reperfusion from arterial clamping[13]. It has been speculated that vascular congestion promotes kidney injury by delaying reperfusion and extending ischemic time to congested areas of the kidney, prolonging the period of tissue hypoxia[14, 15]. Opposing this concept however, there is little evidence of medullary hypoxia in the renal outer-medullary region, or other regions of the kidney, in the initial hours after IR[16, 17]. Rather, greater reductions in filtration and subsequent tubular oxygen demands than that of tissue perfusion, have been found to result in increased or stable tissue oxygen levels during this period[16, 17]. The absence of overt hypoxia in the early reperfusion phase, when severe tubular injury is occurring, brings into question the concept that vascular congestion causes tubular injury by prolonging tissue hypoxia.

The goal of the current study was to determine whether vascular congestion causes tubular injury independent of extending ischemia time. To promote vascular congestion during ischemia we utilized renal venous clamping. During renal venous clamping, the arterial vessels of the kidney remain unobstructed. As such, blood can enter the kidney but is unable to exit due to the venous occlusion. We hypothesized that this would result in marked vascular congestion during the clamp or ischemic period. Conversely, during the ischemic phase of arterial clamping, blood cannot enter the kidney and vascular congestion is limited[13]. Only following reperfusion when blood is again able to enter the kidney, does severe vascular congestion of the renal medulla occur[13]. As such, by comparing tubular injury between arterial and venous clamping without reperfusion, we hoped to be able to examine the effects of vascular congestion on tubular injury independent of extending ischemia time. We hypothesized that vascular congestion during the ischemic phase would cause direct tubular injury.

METHODS

Animals

All experiments were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and were approved and monitored by the Augusta University Institutional Animal Care and Use Committee. Male and female Wistar Kyoto (WKY) rats from Charles River Laboratories were used in all experiments. Rats were housed in temperature (20–26°C) and humidity (30–70%) controlled, 12:12 hour light-cycled conventional animal quarters. Rats were provided ad libitum access to water and standard 18% protein rodent chow (Envigo Teklad, 2918).

Warm bilateral ischemia-reperfusion surgery and tissue harvest

Ischemia-reperfusion was performed as previously described[13]. Briefly, animals were anesthetized with ~3% isoflurane and 95% oxygen. Body temperature was maintained at ~37°C for the duration of the surgery by servo-controlled heating table and infrared heat lamp (R40, Satco S4998). The renal pedicles were accessed via left and right dorsal flank incisions and blunt dissection with cotton tip applicators. The renal artery was then separated from the renal vein for each kidney using curved forceps. For each animal, the renal artery for one kidney and renal vein for the other kidney were clamped with Schwartz Micro Serrefines (Fine Science Tools #18052-03, Foster City, CA) for either 15 or 45 minutes. The kidney which received artery or vein occlusion was rotated for each animal. At the end of the clamp period, the kidneys were excised prior to removal of the vascular clamp. The kidneys were fixed (VIP-Fixative, Fisher #23-730-587) for histological analysis.

In animals shown in **Figure 9**, warm bilateral ischemia reperfusion was performed, and animals allowed to recover for either 0 (no reperfusion), 1, 2, 6, 10 or 24 hours prior to harvesting the kidney for histological analysis, as previously reported[13]. Corresponding injury and congested data is reported in McLarnon et al[13].

Blood free perfusion studies

Animals were anesthetized with ~3% isoflurane and 95% oxygen. Body temperature was maintained at ~37°C for the duration of the surgery by servo-controlled heating table and infrared heat lamp. A midline incision was performed, and the abdominal aorta cannulated. The mesenteric artery and vessels of the right kidney were ligated, and a loose tie placed around the abdominal aorta superior to the renal arteries. The renal vein was separated from the renal artery close to its origin to allow clamp placement. The tie around the aorta was then retracted to prevent blood flow to the left kidney and the kidney flushed of blood via retrograde perfusion of the abdominal aorta using warm ~37°C saline at ~120mmHg using a syringe heater (EGHSL10, New Era Pump Systems, Farmingdale, NY, USA) and pressure gauge. In blood free animals, the renal vein was clamped during the saline perfusion and the abdominal aorta above the kidney ligated to prevent blood reperfusion. Renal perfusion pressure was maintained at ~120mmHg for 5 minutes by adjusting syringe pressure before clamping the renal pedicle for the remaining 40 minutes of ischemia. In control blood perfused kidneys, the kidney was flushed with warm saline but allowed to re-perfuse with blood before clamping the renal vein. Again, the renal pedicle was clamped following 5 minutes of perfusion. At the end of the occlusion period, the kidneys were excised prior to removal of the vascular clamp. The kidneys were then decapsulated, bisected and fixed for histological analysis.

Assessment of vascular congestion

Paraffin-embedded kidney sections were stained with Gomori's trichrome (Thermo Scientific, Cat. No.87020), according to the manufacturer's instructions. Congestion of outer-medulla (OM) vasa recta

(VR) and OM plexus capillaries was assessed using a semi-quantitative scoring method as previously described[13]. Briefly, a score of 0-5 was given to reflect the extent of RBC congestion independently in each region. A score of 0 represents conditions in which all vessels appear open (0% congestion) and a score of 5 represents congestion in all vessels visualized (100% congestion). Scale: 0 = 0%, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, and 5 = 100%. All scoring was done by a researcher unaware of the group identifiers of the samples being scored.

Assessment of tubular injury

Trichrome stained kidney sections were scored for tubular injury (% injured tubular cells) by a pathologist blinded to the hypothesis of the study and sample identifiers. Tubular injury (cell swelling/necrosis) and tubular cast formation were scored in both the cortex and outer medulla (OM). Tubular injury and cast formation is reported as a score of 0-5 with a score of 0 indicating 0-5%, 1, indicating 5-20%, 2, indicating 20-40%, 3, indicating 40-60%, 4, indicating 60-80%, and 5, indicating 80-100% % of all tubules demonstrating that trait.

Assessment of red blood cell uptake by renal tubules

To quantify RBC uptake into tubular cells, 3 non-overlapping 100X images of trichrome stained sections of each of the renal cortex, outer-stripe of the outer-medulla, inner-stripe of the outer-medulla and papilla were taken using an BX40 microscope connected to a DP72 camera (Olympus Imaging Corp, Tokyo, Japan) using an oil immersion lens and Cell Sens imaging software (Olympus imaging Corp, Tokyo, Japan). To avoid selection bias, images were taken by a researcher unaware of the hypothesis being tested or the source of the samples. Images were then viewed with Metamorph imaging software (Molecular Devices, LLC, San Jose, CA) and each cell within an image scored to have either 1) no evidence of RBC uptake, 2) some evidence of RBC uptake, 3) moderate RBC uptake, 4) severe uptake (a tubular cell laden with RBCs). Cells were identified by a nucleus. For each animal, the percentage of cells in each region of the kidney demonstrating either moderate or severe RBC uptake was then calculated.

Multiphoton imaging

Animals were anesthetized with ~3% isoflurane and 95% oxygen. Body temperature was maintained at ~37°C for the duration of the surgery by servo-controlled heating table and infrared heat lamp. Approximately 10mL of whole blood was withdrawn from a donor animal of the same, strain, age and sex into an EDTA coated tube to prevent clotting. RBCs were pelleted and the plasma and buffy coat removed. The RBC pellet was then resuspended in 10mL of saline containing 150ug BCECF-AM (ThermoFisher Scientific, Greenville, NC, USA) in 50uL of dimethylsulfoxide (DMSO). RBC were loaded with dye on ice for 45 minutes before being pelleted, the supernatant removed and replaced with 40mL of saline. This wash step was then repeated 2 more times before RBC were resuspended in warm (~37°C) saline to a total volume of 10mL. Recipient animals were surgically prepared as per blood free perfusion studies. The kidney was then perfused with BCECF labelled RBC before clamping the renal vein for 45 minutes. Following the period of clamping, the left kidney was then removed and a ~5mm section cut and glued to a petri dish. The dish was then filled with saline solution to prevent the tissue from drying. A section from the excised right kidney was utilized as a control for auto-fluorescence. Tissue slices were then imaged using a Bruker 2P-Plus two-photon microscope (Bruker, USA) at 950nm excitation with a 25x objective (N.A. 1.1).

Transmission Electron Microscopy

Preparation and imaging of kidney tissue was performed by the Augusta University Histology Core facility. Tissue was fixed in 4% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate

(NaCac) buffer, pH 7.4, postfixed in 2% osmium tetroxide in NaCac, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series, and embedded in Epon-Araldite resin. Thin 75 nanometer sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Bannockburn, IL), collected on copper grids, and stained with uranyl acetate and lead citrate. Tissue was observed in a JEM 1400 Flash transmission electron microscope (JEOL USA, Peabody, MA) at 120 kV and imaged with a Gatan 1095 OneView camera (Gatan, Pleasanton, CA).

RESULTS

Renal venous clamping results in increased vascular congestion in the cortex than renal artery clamping. To investigate the mechanism(s) through which vascular congestion causes tubular injury, we first compared vascular congestion induced by occlusion of either the renal artery or the renal vein for both 15 and 45 minutes, without reperfusion, in male and female WKY rats (**Figure 1 A-B**). There were no significant sex differences in the degree of vascular congestion between male or female rats with either arterial or venous clamping. While moderate vascular congestion was present in the cortical peritubular capillaries and veins following 45 minutes of ischemia from both renal arterial and venous clamping, it was significantly greater following venous clamping compared to arterial clamping (**Figure 1.B, P=0.02**).

Renal venous clamping results in greater vascular congestion in the outer-medullary plexus than does renal artery clamping. We found that renal venous occlusion resulted in significantly greater OM plexus congestion following both 15 (**Figure 1.C, P<0.0001**) and 45 minutes (**Figure 1.D, P<0.0001**) of clamping. There was severe congestion in the OM plexus following 45 minutes of venous clamping while OM plexus congestion was minimal following arterial clamping. The larger vasa-recta vessels were congested following both arterial and venous clamping, however vasa recta congestion was more prominent following venous clamping. There were no differences in congestion in the outer-medulla between male and female rats.

Renal venous clamping results in marked tubular injury in the renal outer-medulla whereas with arterial clamping outer-medullary tubular injury was minimal. Following as little as 15 minutes of renal venous clamping without reperfusion, there was significant tubular injury in the renal outer-medulla, including cell swelling and tubular cast formation. In contrast, few tubules in the outer-medullary region demonstrated significant tubular injury following 15 minutes of arterial clamping (**Figure 2.A, P<0.0001**). Tubular injury in the outer-medulla was increased following 45 minutes of venous clamping with marked tubular necrosis and loss of tubular structure observed in this region (**Figure 2.H**). In contrast, tubular tubular injury remained low and tubular morphology looked relatively normal following 45 minutes of arterial clamping without reperfusion (**Figure 2.B and F**). There were no differences in tubular injury between male and female rats at either time point. Tubular cast formation in the renal outer-medulla was limited to kidneys in which the renal vein was clamped (**Figure 2.C-D**). There were no sex differences in cast formation in the outer-medulla following either 15 or 45 minutes of arterial or venous clamping (**Figure 2.C-D**).

Despite the same clamp time, renal venous clamping results in greater cortical tubular injury compared to renal artery clamping. Following 15 minutes of clamping without reperfusion there was significant evidence of tubular injury in the kdiney cortex, however, this was greater with renal venous compared to renal arterial clamping (**Figure 3.A, P=0.003**). In both males and females, all of the tubules in the renal cortex demonstrated tubular injury following 15 minutes of venous clamping. At this same time point, only approximately 60% of cortical tubules from kidneys with an arterial clamp were injured. Cortical tubular injury was increased in kidneys with arterial clamping following 45 minutes of ischemia when compared to 15 minutes of ischemia, however, the % of injured tubules remained significantly

less than that of kdineys with venous clamping (**Figure 3.B, P=0.003**). There were no differences in tubular injury between male and female rats at either time point. Tubular cast formation in the renal cortex was again limited to animals in which the renal vein was clamped (**Figure 3.C-D**). Casts appeared more developed following 45 minutes of venous clamping when compared to 15 minutes oof clamping. Following 15 minutes of venous clamping, casts often appeared as discrete droplets within the tubular lumen (**Figure 3.G**), rather than a continuous cast. There were no sex differences in cast formation in the kidney cortex following either 15 or 45 minutes of arterial or venous clamping (**Figure 3.C-D**).

Tubular cells appeared to take up RBCs and this was enhanced by venous clamping. High power trichrome stained sections revealed the presence of numerous red droplets within tubular cells. These were present in cortical tubules following both arterial and venous clamping but were most prominent following venous clamping for 45 minutes (**Figure 4.A & B and E**). These red droplets were also prominent in injured cells or tubules. Some cells appeared so laden with red droplets that they appeared to turn bright red and were almost indistinguishable in color from RBC in surrounding vasculature (**Figure 4.A and 4.1**). These red droplets were less prominent in the inner-stripe of the outer-medulla, being primarily observed in injured tubules following 45 minutes of venous clamping (**Figure 4.C**). Droplets were almost completely absent in tubules of the inner-stripe of the outer-medulla following arterial clamping (**Figure 4.D**). There were no sex differences in the tubular density of these red droplets, as assessed in trichrome sections (**Figure 4.E**).

Electron microscopy imaging of the kidney following 45 minutes of venous clamping demonstrates that RBCs are being taken up by tubular cells and their contents secreted into the tubular lumen. Electron microscopy images from the kidneys of rats following 45 minutes of renal venous clamping without reperfusion identified electron dense components that appeared to be RBCs within tubular cells (**Figure 5.A**). These RBC-like structures within tubular cells often appeared to be in difference states of decay, with varying electron density often observed (**Figure 5.B**). Similar electron dense material was often observed within the tubular lumen, suggesting it was being secreted by the tubular cells (**Figure 5.A-C**). Confirming these electron dense structures were RBCs, these structures were completely absence from sections, and the lumen clear of electron dense material, from blood free kidneys following 45 minutes of venous clamping (**Figure 5.D**).

Tubular cells become fluorescent following venous clamping with dye loaded RBCs. Following perfusion of the kidney with RBCs labelled with a cell permanent fluorescent dye and 45 minutes of venous clamping without reperfusion, many, but not all tubules, demonstrated significant fluorescence (**Figure 6.A**). In some tubules, fluorescent material appeared to fill the lumen, consistent with secretion of the dye (**Figure 6.B**). At the same power, tubular fluorescence was minimal in kidneys not perfused with dye loaded RBC (**Figure 6.C**).

Evidence of tubular phagocytosis of RBC and uptake of degraded RBC material between in invaginations of the basolateral membrane. In some images from kidneys following 45 minutes of venous clamping, the basolateral membrane of tubular cells was found to ruffle or closely wrap around what appeared to be intact extravasated RBC in the interstitial space (**Figure 7, A-B**). This suggesting phagocytosis of whole RBC may be occurring across the basolateral membrane of tubular cells. Electron dense material from degraded RBCs was also commonly observed between the folds or invaginations of the basement membrane and appeared to be being secreted through these invaginations into the tubular lumen (**Figure 7, C-D**)

Injury from venous clamping is minimal in the blood free kidney. To determine whether tubular injury from venous clamping required the presence of blood, we compared tubular injury between blood

perfused kidneys and saline flushed kidneys that were blood free following 45 minutes of venous clamping without reperfusion. Vascular congestion and evidence of tubular RBC uptake were significantly greater in the control blood perfused kidney than in saline flushed kidneys following 45 minutes of ischemia in both the cortex (**Figure 8.A**) and outer-medulla (**Figure 8.B**). Tubular cast formation was exclusive to the blood perfused kidney (**Figure 8.A-B**)..

There is evidence that mass tubular uptake of RBCs also occurs following reperfusion from arterial clamping. Vascular congestion of the kidney cortex and venous circulation occurs during periods of ischemia and marked vascular congestion of the outer medulla occurs following reperfusion from arterial clamping. Further, tubular injury is known to promote later renal vasoconstriction which may result in generalized kidney congestion. To determine if RBC uptake occurs following reperfusion from arterial clamping, we quantified RBC uptake prior to removing the clamp, and at time points of 1, 2, 6, 10 and 24 hours post removal of the clamp, following 45 minutes of warm bilateral ischemia from arterial clamping. At 24 hours post-reperfusion from arterial clamping, proteinaceous casts and tubular swelling was prominent. This was associated with histological evidence of RBC uptake like that observed following venous clamping without reperfusion, including many bright red 'RBC laden' tubular cells and red droplets within swollen tubular cells (**Figure 9 A-C**). Bright red tubular cells often appeared necrotic and had detached from the basement membrane. Quantification of RBC uptake over time revealed that RBC uptake increased in the more superficial regions of the kidney over the initial 24 hours of the reperfusion period from arterial clamping (**Figure 9.D**). By 24 hours post-reperfusion, as many as 40% of tubular cells in the outer-stripe of the outer-medulla and 30% of cortical tubule cells displayed significant RBC uptake. RBC uptake in the inner-stripe of the outer-medulla was less prominent and was maximal by 2 hours post-reperfusion but remained elevated through 24 hours (**Figure 9.D**). A similar pattern was observed in collecting duct cells of the papilla, however, maximal uptake of RBC was not achieved until 6 hours post-reperfusion (**Figure 9.D**).

DISCUSSION

This study has two major findings: 1) That vascular congestion results in rapid extravasation and uptake of RBC by the kidney tubules on mass in both the cortex and medulla and 2), that this results in toxic injury to the tubular cells.

As expected, we found that renal ischemia induced by renal venous clamping resulted in greater vascular congestion than renal arterial clamping, particularly in the OM peritubular capillary networks. Greater vascular congestion in the venous clamp model was associated with significantly greater tubular injury, including cell swelling and luminal cast formation in the OM as early as 15 minutes into the clamp period. By the end of the 45-minute ischemic period marked tubular necrosis was observed in the outer-medulla along with significant tubular injury and cast formation in the cortex. Demonstrating that this injury was not due to ischemic time alone, despite the same or potentially reduced ischemia time, tubular injury was markedly less following 45 minutes of arterial clamping compared to venous clamping. High power trichrome stained sections revealed the presence of numerous red droplets within tubular cells, which were most prominent following venous clamping and in severely injured cells. Electron microscopy images identified these droplets as RBCs and indicated that RBCs from congested capillaries were being extravasated and taken up by tubular cells. While the mechanisms underlying RBC extravasation and uptake remain unclear at present, the basolateral membrane of tubular cells could often be seen to wrap around intact RBCs, suggesting phagocytosis plays a role. Phagocytosis of intact erythrocytes by tubular cells has previously been reported in both rats[18] and humans[19] with hematuria, however, to the best of our knowledge, this is the first report of uptake of RBC's from the capillary network across the basolateral membrane. Electron dense material from what appeared to be degraded RBCs was also commonly observed to be taken up between the folds or invaginations of the

basement membrane of proximal tubular cells and appeared to be being secreted through these invaginations into the tubular lumen. The electron dense, proteinaceous material in the lumen could not have been due to glomerular damage and greater protein leak from the blood as, despite the absence of filtration, it worsened over the ischemic period. Further, following 15 minutes of venous clamping, small bubbles of cast material could be observed which appeared to be being secreted directly from tubular cells (**Fig 3.G**). Labelling of RBCs with a fluorescent dye confirmed the mass extravasation and uptake of RBCs by tubular cells during ischemia caused by venous clamping. As RBC's were loaded with dye *ex vivo* and washed to remove excess dye prior to re-introduction to the animal, the increased tubular fluorescence in animals given dye loaded RBCs could only have come from tubular uptake of RBCs or their components. Dye was also found within the tubular lumen, supporting basolateral to apical transport and secretion. To confirm that tubular injury during ischemia from venous clamping was due to the presence of vascular congestion and RBC uptake, we then compared venous clamping between blood and saline perfused, blood free kidneys. Despite the same clamp time, tubular injury was significantly greater, and tubular cast formation limited to- the blood perfused kidney.

Grutzendler *et al* recently reported that recanalization of small vessels occluded by emboli occurs by extravasation across the capillary wall in several organs including the kidney[20]. In the kidney, these extravasated emboli are then engulfed by tubular cells[20]. Grutzendler *et al* speculate that this 'angiophagy' of emboli, represents and alternative mechanism to hemodynamic forces and the fibrinolytic system to clear obstructions from the microcirculation[20]. Critically, our novel data provide the first evidence that similar processes occur on mass in the kidney within minutes of vascular congestion forming. While this may be an attempt to help re-cannulate micro vessels congested with packed RBC, it appears that the mass extravasation and prolonged uptake of RBCs during periods of congestion overwhelms the tubules, leading to toxic injury. Madsen et al previously reported toxic injury to proximal tubules from the luminal uptake of RBCs, including loss of brush boarder, increased lysosome-like bodies, lipid accumulation, necrosis and a later inflammatory response[18]. IN our studies, the tubules of the outer medulla appear to be most vulnerable to RBC toxicity, with many demonstrating severe injury and tubular necrosis despite only modest histological evidence of RBC uptake. Tubules in the cortex appeared to be more resilient, with tubular injury only becoming prominent once cells had become visibly laden with RBCs. Given the renal medulla is susceptible to severe vascular congestion following periods of ischemia, our data provide a novel explanation for the susceptibility of the renal medulla to ischemic injury.

Clinical reports from partial nephrectomy surgery have suggested that occlusion of the renal vein initiates greater renal damage than occlusion of the renal artery[21]. Studies from rodent models have also shown venous clamping is more detrimental than arterial clamping[3-7]. The pathophysiology underlying worsening renal injury following venous clamping when compared to arterial clamping, however, remains unclear. With venous clamping, blood cannot exit the kidney via the renal vein, however, early in the clamp period, oxygenated blood can still enter the kidney via the artery. This results in packing of the renal vasculature with RBC. This continues until intra-renal pressures become so much that blood flow becomes stagnant. Importantly, as the venous clamped kidney still receives some oxygenated blood early in the clamp period, if ischemia alone was the cause of tubular injury, the arterial clamp would have been expected to result in greater tubular injury than the venous clamp. This, however, is not consistent with the data we obtained. An alternative explanation for greater injury in the venous clamped kidney is that injury is driven by the increased intra-renal pressures. Wei *et al* recently reported that increased proximal tubular pressure during the ischemic phase exacerbates kidney injury during venous clamping[22]. While we cannot exclude a role of increased intra-renal pressures in driving RBC uptake and tubular toxicity, the absence of tubular cast formation and RBC laden necrotic cells in saline perfused, venous clamped kidneys, suggests increased pressures alone cannot account for the

tubular injury observed during venous clamping. Rather, our data indicate that greater vascular congestion and RBC mediated tubular toxicity is responsible for renal injury following venous compared to arterial clamping.

RBC uptake and toxicity may be a common mediator of tubular injury in ischemic AKI. Our data suggest that RBC toxicity is not limited to ischemic AKI from venous clamping. Phenotypically similar injury is observed following reperfusion from arterial clamping, including proteinaceous luminal casts and necrotic RBC laden cells[13, 23, 24]. While early in reperfusion, this injury is most prominent in the renal outer-medulla where congestion is greatest[13], our data suggest that RBC uptake and tubular RBC toxicity may also account for injury to more superficial nephrons that develops later in reperfusion. Cortical tubular RBC uptake appears to increase overtime during reperfusion from arterial clamping. We speculate that RBC uptake in cortical tubules occurs both during the ischemic period itself as well as later during reperfusion. This later uptake is likely secondary to injury mediated vasoconstriction that results in stagnation of flow in areas of the cortical circulation[1, 25, 26]. RBC uptake and toxicity as a common mechanism of injury in ischemic AKI explains both the temporal and anatomical patterns of injury observed in the kidney following arterial clamping[2]. RBC toxicity also provides an explanation for the association between tubular injury and vascular congestion early during reperfusion, despite the absence of tissue hypoxia during the same period[13, 16, 17]. A toxic mechanism of injury mediated by release of RBC contents, such as hemoglobin or free iron, within tubular cells would also be consistent with evidence that reactive oxygen species contribute to tubular injury and reports of a protective effect of heme-oxygenase[27-29]. While little is known regarding RBC uptake in human kidneys, RBC like droplets can be observed in necrotic tubular cells following delayed graft function in trichrome stained sections, suggesting similar processes are occurring[30].

In conclusion, our data, for the first time, demonstrate that RBC congestion of the kidney vasculature results in the mass extravasation and uptake of RBC by tubular cells. While this process may have a physiological role to restore perfusion to occluded capillaries, the continued uptake of RBCs by most tubular cells during periods prolonged or severe vascular congestion, appears to overwhelm this process, resulting in toxic injury to the tubules. The basolateral uptake of RBCs and RBC toxicity appear to be a major component of tubular injury in ischemic AKI which has not previously been recognized. RBC uptake and RBC toxicity as a primary mechanism of tubular injury in AKI likely explains the greater injury following venous rather than arterial clamping, the relationship between prolonged periods of low renal perfusion and kidney injury[31] and the failure of diuretics targeting kidney hypoxia to improve outcomes in ischemic AKI[2]. Approaches to slow or prevent RBC extravasation and tubular uptake may provide a novel therapeutic pathway to limit kidney injury in ischemic AKI.

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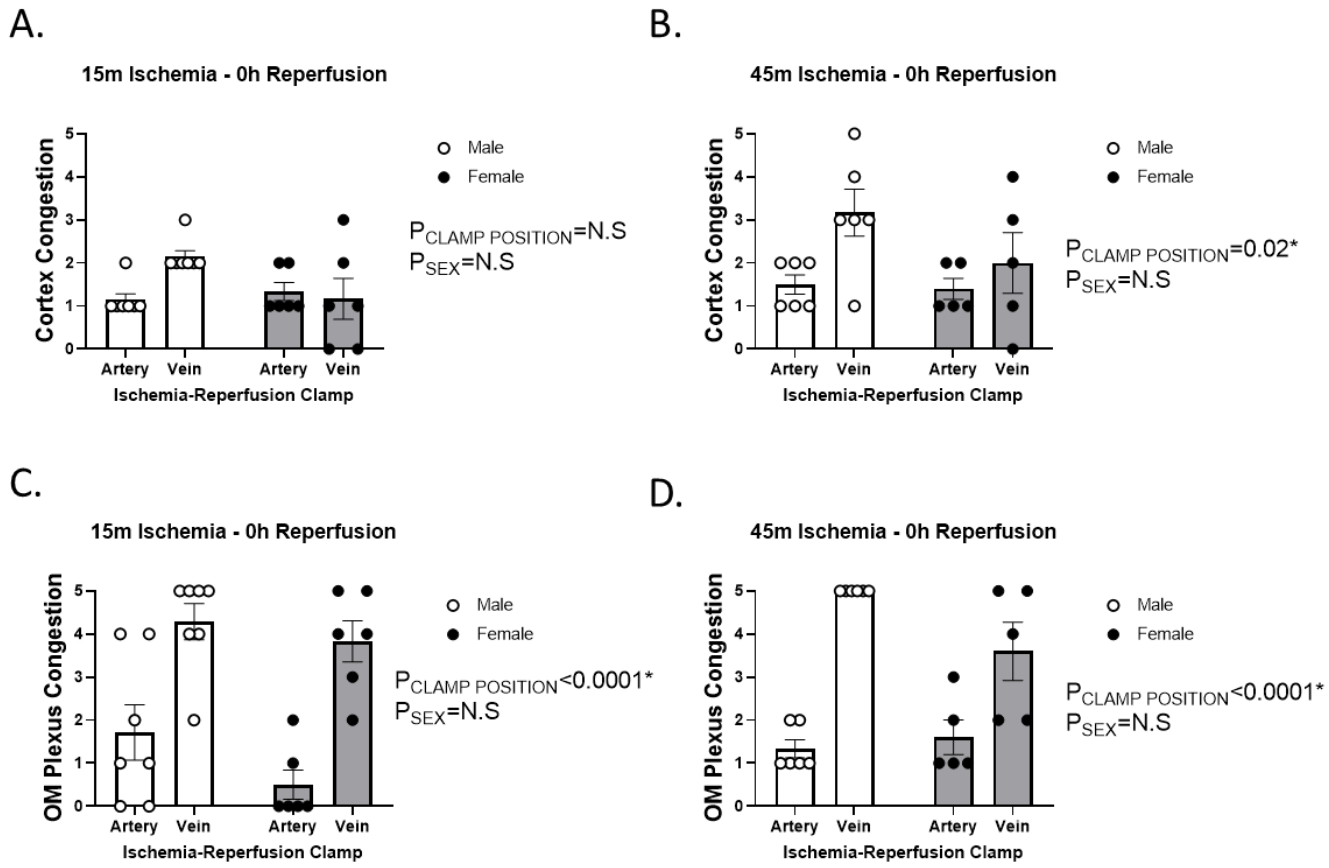

Figure 1. <u>The effect of arterial verses venous clamping on vascular congestion in the cortex and outer-medulla.</u> Vascular congestion scores of the cortex or outer-medullary capillary plexus following 15 (**Panel A and B**, respectively) or 45 minute (**Panel C and D**, respectively) occlusion of the renal artery or renal vein with no reperfusion in male (n=7, white bars) and female (n=6, gray bars) WKY rats. Vascular congestion scores: 0 = 0%, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, 5 = 100%. Values are expressed as mean ± SEM. Two-way ANOVA comparing clamp position and sex, *p<0.05. Note: for each rat, the renal vein was clamped on one kidney and the renal artery was clamped on the other kidney. **Panel E**, Representative images of trichrome stained sections of the cortex following 45-minute occlusion of the renal artery or renal vein with no reperfusion in male and female WKY rats. **Panel F**, Representative images of trichrome stained sections of the outer medulla (OM) following 45-minute occlusion of the renal artery or renal vein with no reperfusion in male and female WKY rats. Images were taken at 20x. Scale bar denotes 50µm.

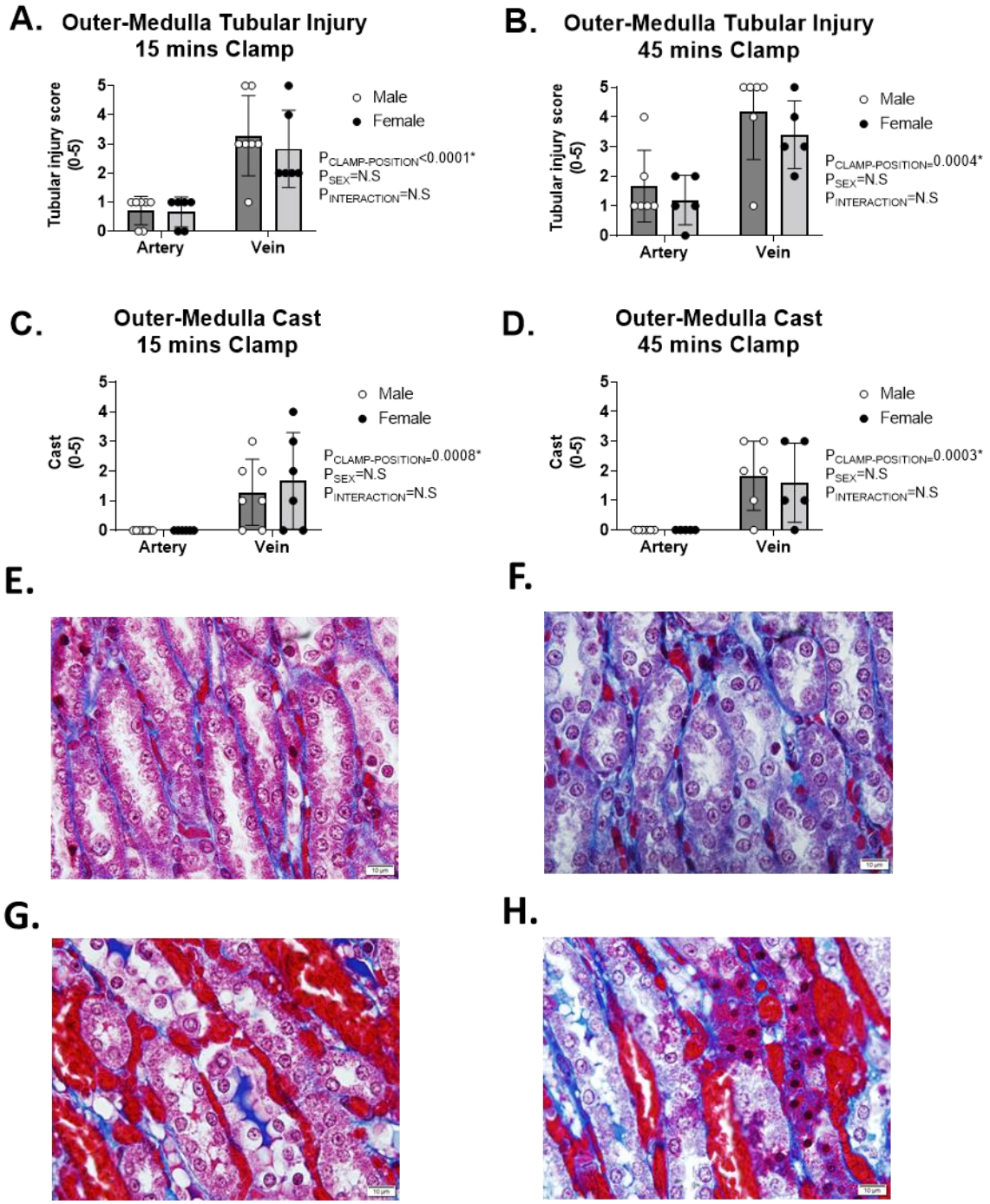

Figure 2. <u>The effect of arterial verses venous clamping on outer-medullary tubular injury.</u> Tubular injury scores of the outer medulla following 15 (**Panel A**) or 45 minutes (**Panel B**), respectively) of occlusion of the renal artery or renal vein with no reperfusion in male (n=7, white circles) and female (n=6, black circles) WKY rats. Tubular cast formation scores of outer medulla following 15 (**Panel C**) or 45 minute (**Panel D**), respectively) occlusion of the renal artery or renal vein with no reperfusion. Tubular injury or cast formation is reported as 0-5, representing the % of tubules demonstrating tubular injury with a score of 0 indicating 0-5%, 1, indicating 5-20%, 2, indicating 20-40%, 3, indicating 40-60%, 4, indicating 60-80%, and 5, indicating 80-100% of all tubules demonstrating that trait. Values are expressed as mean ± SEM. Two-way ANOVA comparing clamp position and sex, *p<0.05. Note: for each rat, the renal vein was clamped on one kidney and the renal artery was clamped on the other kidney.

Panel E-F Representative images of tubular morphology in trichrome stained sections of the outer medulla (OM) following 15 and 45-minutes of occlusion of the renal artery with no reperfusion, respectively. **Panel G-H**, Representative images of tubular morphology in trichrome stained sections of the outer medulla (OM) following 15 and 45-minutes of occlusion of the renal vein with no reperfusion, respectively.

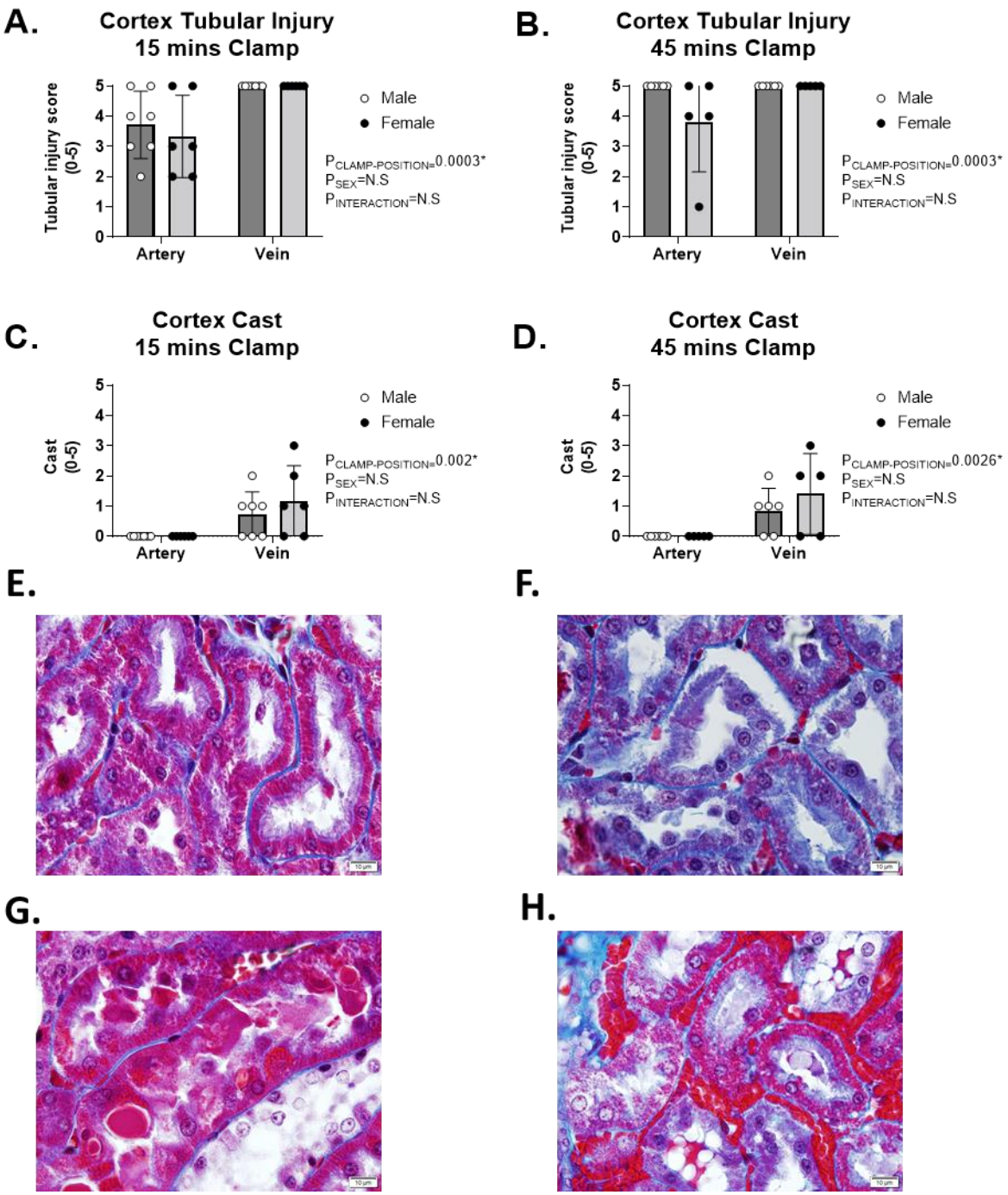

Figure 3. <u>The effect of arterial verses venous clamping on cortical tubular injury.</u> Tubular injury scores of the kidney cortex following 15 (**Panel A**) or 45 minutes (**Panel B**), respectively) of occlusion of the renal artery or renal vein with no reperfusion in male (n=7, white circles) and female (n=6, black circles) WKY rats. Tubular cast formation scores of outer medulla following 15 (**Panel C**) or 45 minute (**Panel D**), respectively) occlusion of the renal artery or renal vein with no reperfusion. Tubular injury or cast formation is reported as 0-5, representing the % of tubules demonstrating tubular injury with a score of 0 indicating 0-5%, 1, indicating 5-20%, 2, indicating 20-40%, 3, indicating 40-60%, 4, indicating 60-80%, and 5, indicating 80-100% of all tubules demonstrating that trait. Values are expressed as mean ± SEM. Two-way ANOVA comparing clamp position and sex, *$p<0.05$. Note: for each rat, the renal vein was clamped on one kidney and the renal artery was clamped on the other kidney.

Panel E-F Representative images of tubular morphology in trichrome stained sections of the cortex following 15 and 45-minutes of occlusion of the renal artery with no reperfusion, respectively. **Panel G-H**, Representative images of tubular morphology in trichrome stained sections of the cortex following 15 and 45-minutes of occlusion of the renal vein with no reperfusion, respectively.

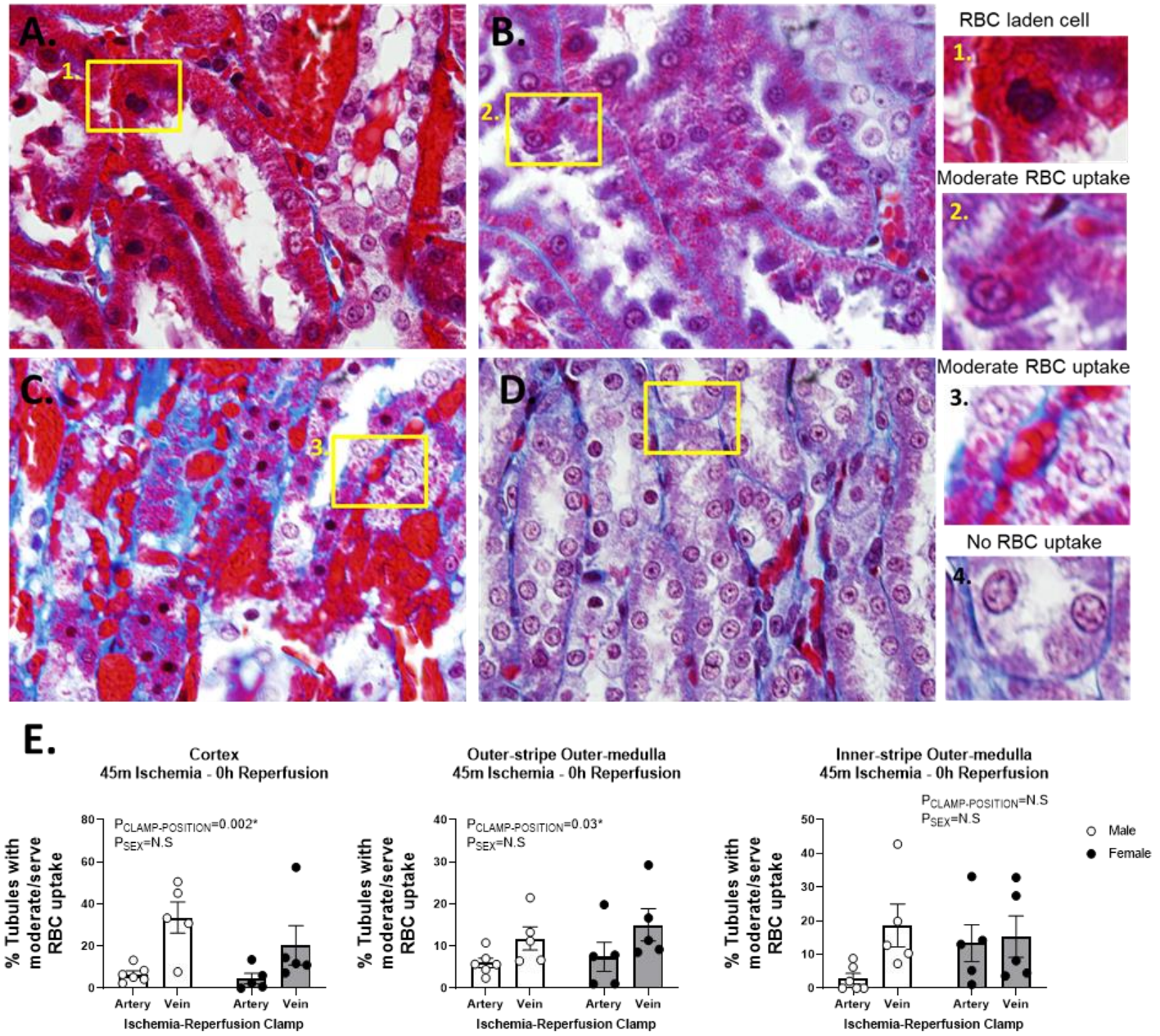

Figure 4 <u>The effect of arterial verses venous clamping on tubular uptake of red blood cells (RBC).</u> **Panel A** Representative image of trichrome stained section of the kidney cortex following 45 minutes of venous occlusion. Red droplets can be observed to have accumulated within the tubular structures, causing them to appear bright red in trichrome stained sections. The yellow box (**1.**) highlights the typical appearance of a RBC laden tubular cell. **Panel B**, Representative image of trichrome stained section of the cortex following 45-minute occlusion of the renal artery with no reperfusion. Red droplets are observed in cortical tubules, however the density of these is less than that following venous clamping and not associated with tubular cast formation. Box (**2.**) highlights the appearance of a typical cortical tubular cell with moderate RBC uptake. Numerous red droplets can be observed within the cytosol. **Panel C** Representative image of trichrome stained section of the kidney outer-medulla following 45 minutes of venous occlusion. Swollen cells are common and red droplets can be observed to have accumulated within the tubular structures. The yellow box (**3.**) highlights the typical appearance of a typical outer-medullary tubular cell with moderate RBC uptake. **Panel D**, Representative image of a trichrome stained section of the outer-medulla following 45-minutes of arterial occlusion with no reperfusion. Red droplets are absent from tubular structures. Box (**4.**) highlights the appearance of a

typical outer-medullary tubular cell without RBC uptake. RBC uptake scores of the cortex, outer-stripe of outer-medulla, inner-stripe of outer-medulla and papilla following 45 minute (**Panel E**) occlusion of the renal artery or renal vein with no reperfusion in male (n=6, white bars) and female (n=5, gray bars) WKY rats. RBC uptake is reported as % tubule cells demonstrating either moderate or severe (RBC laden cells) RBC uptake. Values are expressed as mean ± SEM. Two-way ANOVA comparing clamp position and sex, *$p<0.05$. Note: for each rat, the renal vein was clamped on one kidney and the renal artery was clamped on the other kidney.

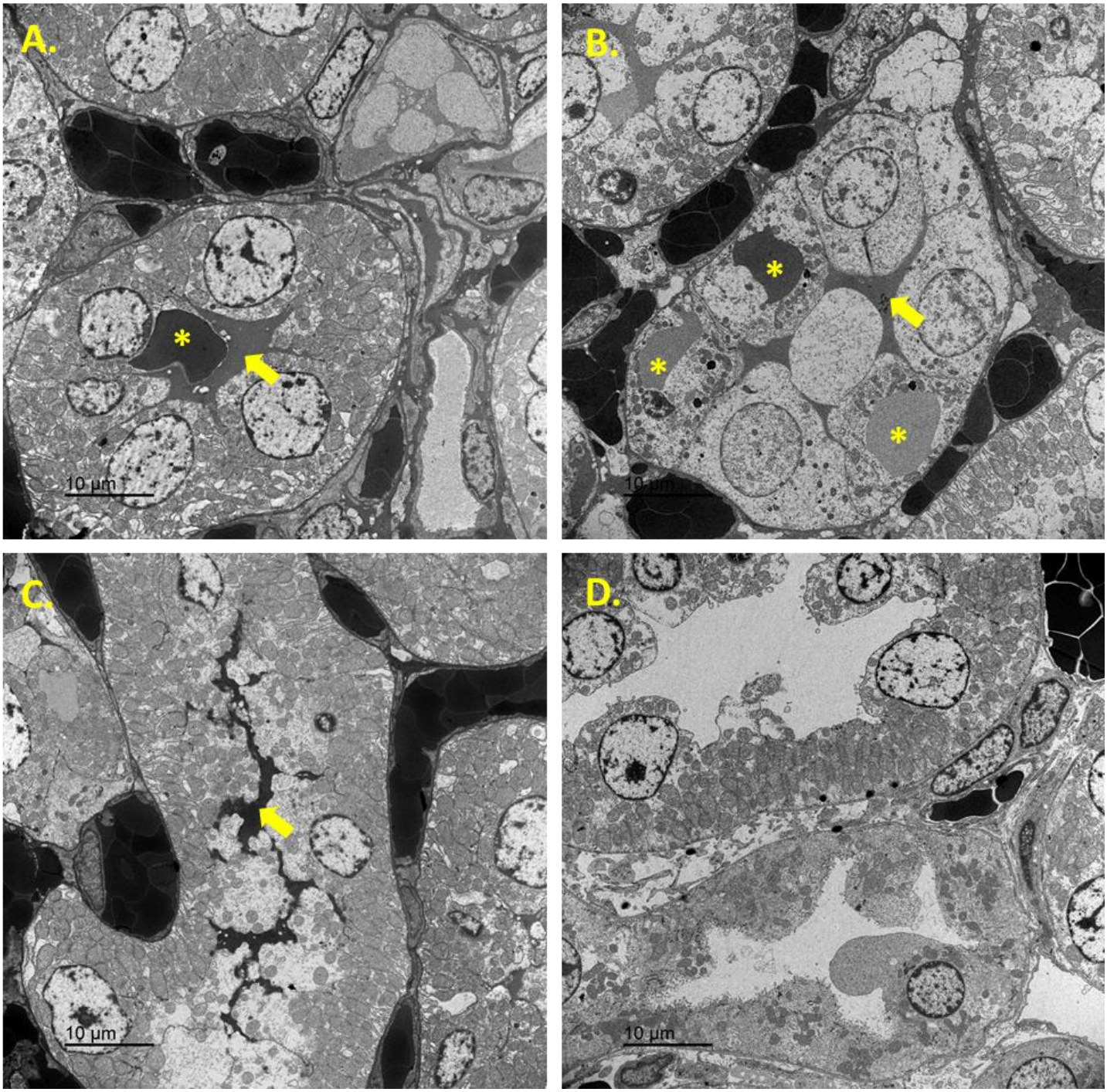

Figure 5. Red blood cells or their components are taken up and secreted by the tubules. **Panels A, B and C** are electron micrographs of the outer-medulla of a rat kidneys 45 minutes after venous clamping. ***** identify RBCs within tubular cells which appear to be in difference states of decay. **Arrows** point toward RBC like material which appears to be secreted into the tubular lumen. Confirming these electron dense structures are RBCs, these are absent and the lumen open in the blood free kidney following the same 45 minutes of venous clamping (**Panel D**).

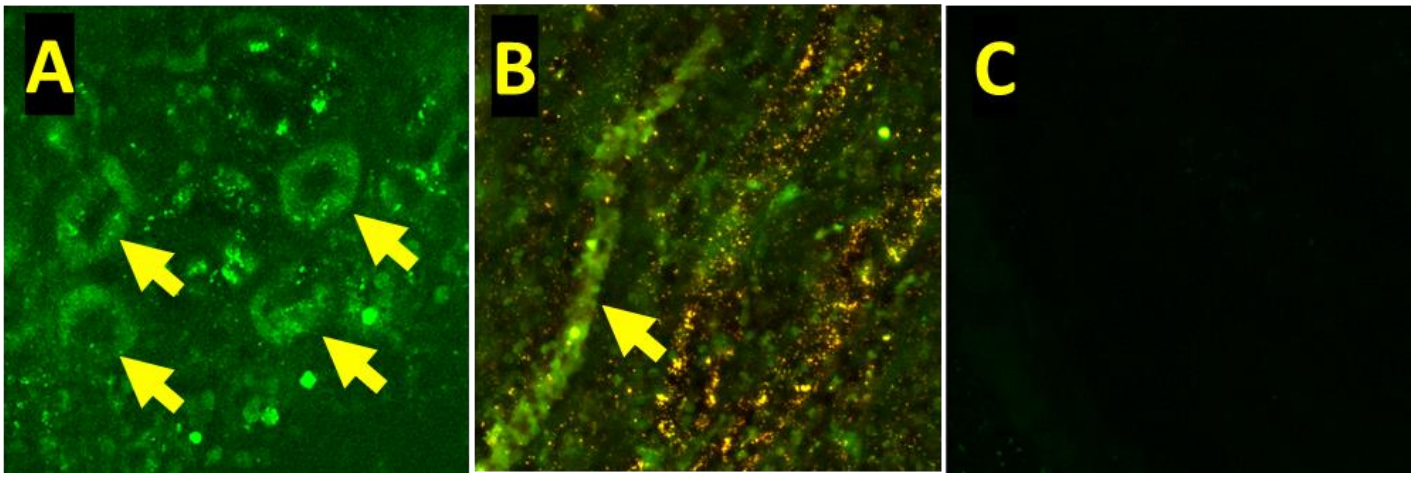

Figure 6. Tubular cells accumulate fluorescent dye from cell permanent dye loaded RBC during venous clamping. RBC uptake was evident as fluorescence (BCECF-AM) from fluorescent dye loaded RBC was present in tubular cells harvested 45 minutes after venous clamping (**A, arrows**). Fluorescent dye could also be observed in the lumen of some tubules (**B, Arrow**), consistent with tubular secretion of RBC contents. Yellow represents red tubular autofluorescence. The fluorescent signal came from the RBC as it was absent in kidneys not loaded with fluorescent RBCs (**C**).

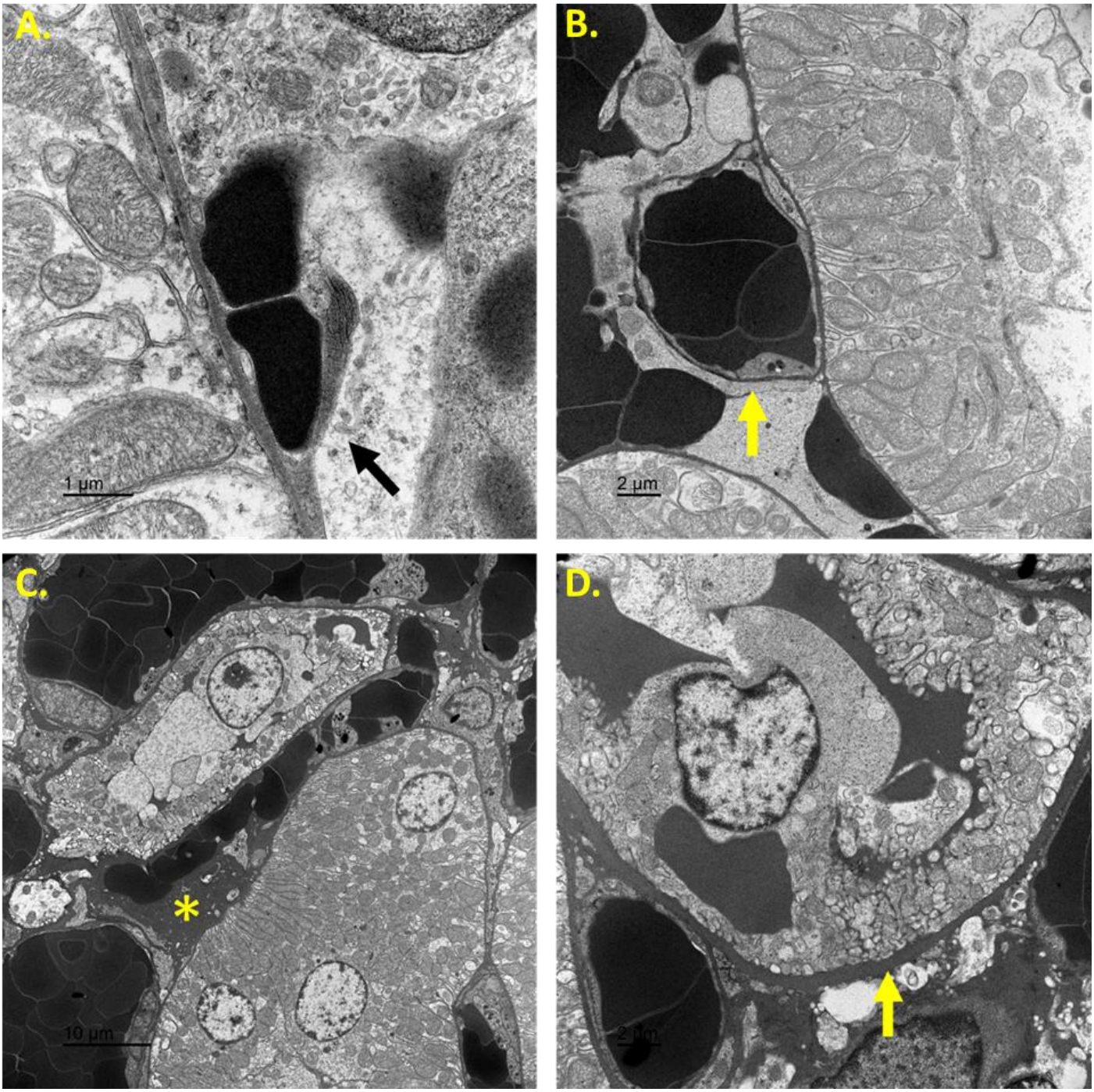

Figure 7. <u>The basolateral membrane of tubular cells was often found to wrap around extravasated RBC and electron dense material could be observed between basolateral membrane invaginations.</u> In electron micrographs of the outer-medulla 45 minutes after clamping the renal vein extravasated RBCs can be observed appearing to be phagocytosed by tubular cells across the basolateral membrane. **Image A**, The black arrow shows the basement membrane of a tubular cell appearing to ruffle and wrap around RBC's (left image). **Image B**, The yellow arrow (right image) shows the basement membrane of a tubular cell which has completely wrapped around a groups of RBCs. Electron dense material from degraded RBCs was also commonly observed to be taken up between the folds or invaginations of the basement membrane and secreted into the tubular lumen. **Image C**, Electron dense material (*) can be seen outside tubular cells and is continuous with darkly stained basolateral membrane invaginations. **Image D**, A higher magnification view showing electron dense material appearing to traverse between the basolateral and apical membrane between membrane invaginations.

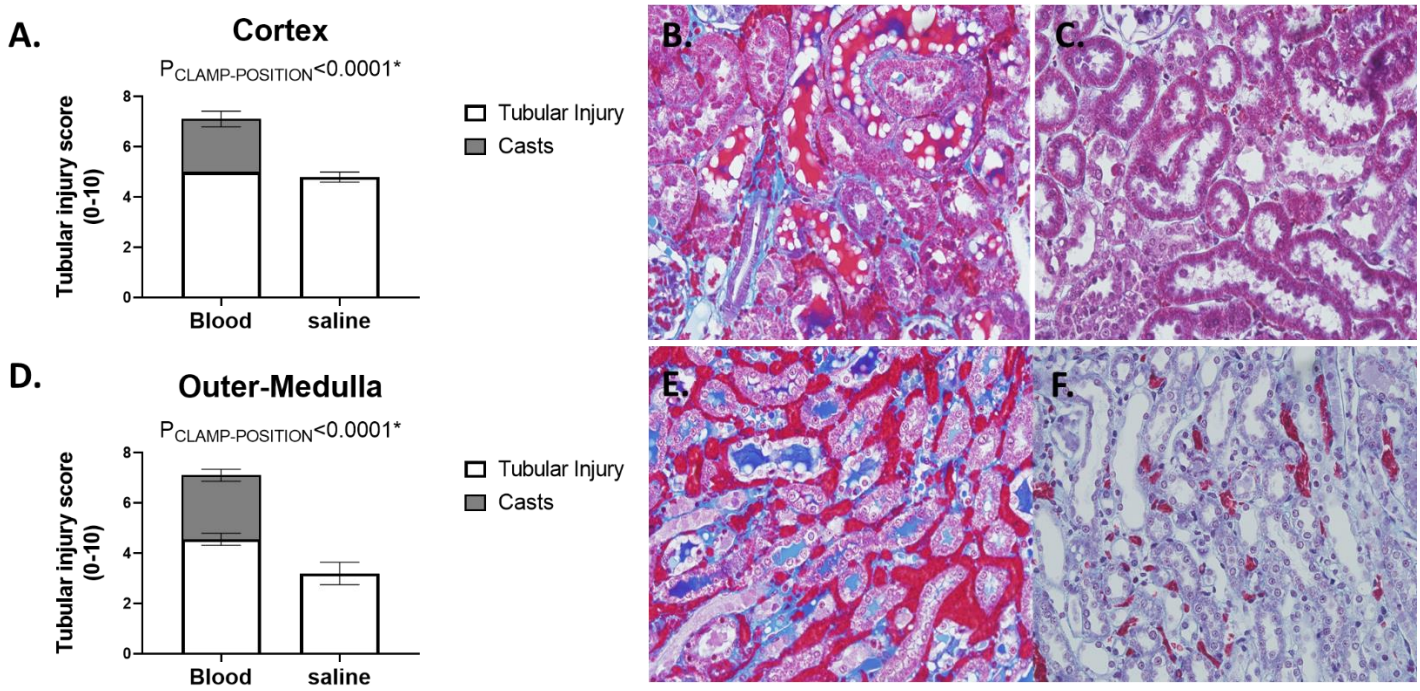

Figure 8. <u>The effect of saline flushing on tubular injury following 45 minutes of venous clamping without reperfusion.</u> **Panel A**, Tubular injury scores of the cortex following 45 minutes of renal venous clamping with no reperfusion in blood and blood free kidneys. **Panel D**, Tubular injury scores of the outer-medulla (OM) following 45 minutes of renal venous clamping with no reperfusion in blood and blood free kidneys. As sex differences were not observed, data from male (n=3-4) and female (n=5) WKY rats were pooled for each panel. Tubular injury is reported as a score of 1-15 with a possible 5 points for each of cell sloughing, tubular injury or cast formation. 0-0-5%, 1-5-20%, 2-20-40%, 3-40-60%, 4-60-80%, 5-80-100% % of all tubules demonstrating the trait. Values are expressed as mean ± SEM. Statistics represent Students t-test comparing clamp position, *p<0.05. Representative images of trichrome stained sections of the cortex following 45 minutes of venous occlusion with no-reperfusion in control (**Panel B**) and blood free (saline flushed kidney; **Panel C**). Representative images of trichrome stained sections of the outer-medulla following 45 minutes of venous occlusion with no-reperfusion in control (**Panel E**) and blood free (saline flushed kidney; **Panel F**). Initial images at 40X magnification. Tubular casts and cell swelling are prominent in the blood perfused kidney but absent in saline flushed kidneys following venous clamping.

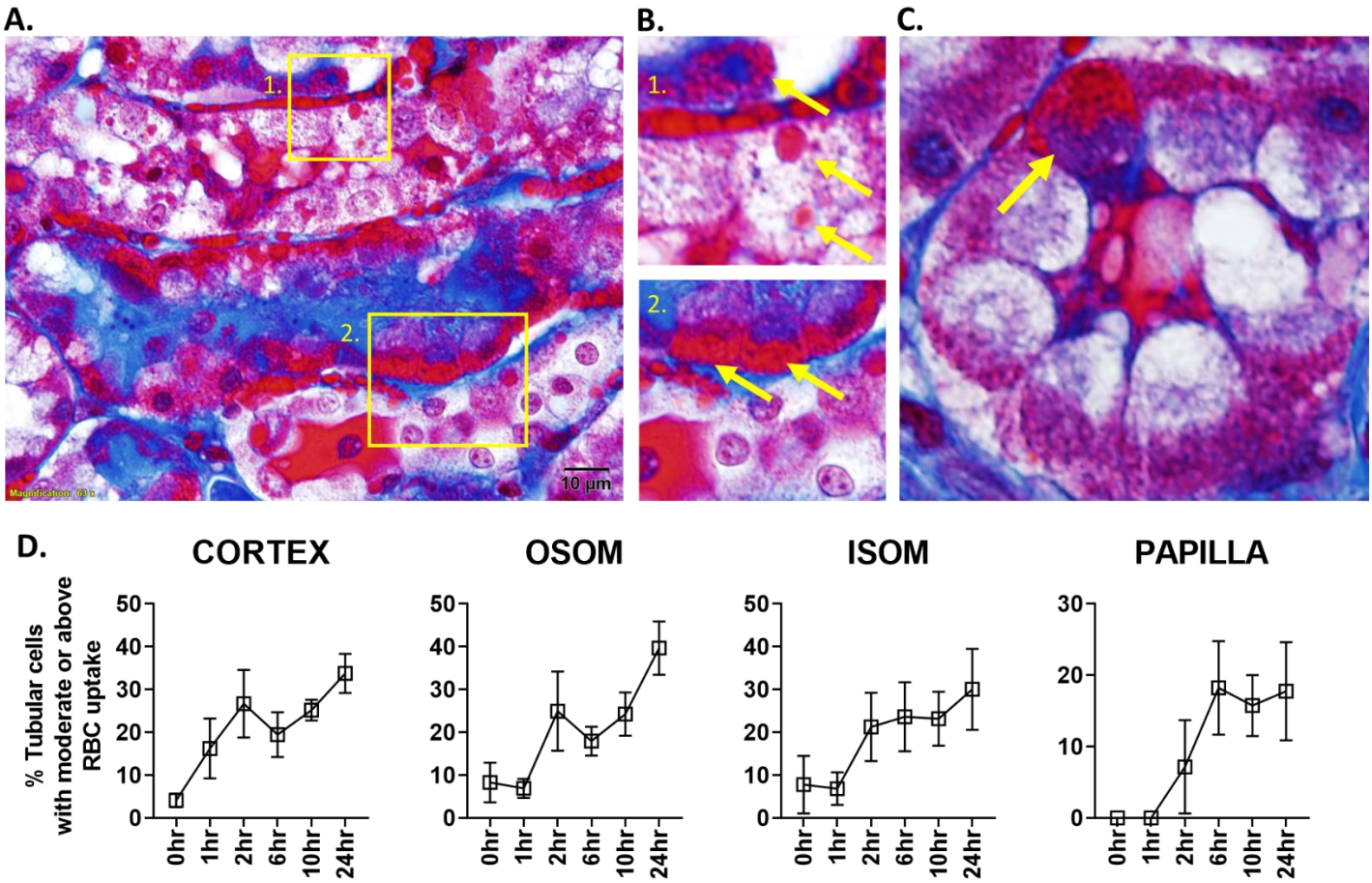

Figure 9. Renal tubular cell uptake of RBCs increases following reperfusion from 45 minutes of warm bilateral arterial clamping. **Panel A** shows a 100X image of the outer-medulla of a rat kidney 24 hours after warm-bilateral IR from arterial clamping. **Panel B** is zoomed images from panel A from the regions depicted in the yellow box labelled (**1.**) and (**2.**) with arrows identifying RBC droplets within tubular cells or RBC laden tubular cells, identified by their bright red trichrome staining. **Panel C** highlights the change in color of a single tubular cell laden with RBC (arrow) compared to other tubular cells in the same structure. In **Panel D**, tubular cells RBC uptake is quantified in the renal cortex, outer-stripe of outer-medulla (OSOM), inner-stripe of outer-medulla (ISOM) and papilla from time=0hr (45 minutes ischemia, no reperfusion) through 1, 2, 6, 10 and 24 hours post reperfusion in male WKY rats. Each time point represents quantification of 4-6 animals. RBC uptake is reported as % tubule cells demonstrating either moderate or severe (RBC laden cells) RBC uptake. Values are expressed as mean ± SEM. Two-way ANOVA comparing clamp position and sex, *$p<0.05$. The percent of tubular cells in all regions demonstrating either moderate or severe RBC uptake increases over the reperfusion period in all regions.