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Research Article

Prolonged Warm Ischemia Time is Associated with Oxidative Stress and Inflammation after Kidney Ischemia-Reperfusion in Rat model

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Abstract

Donation after circulatory death (DCD) kidney transplants inevitably sustain a degree of warm ischemic (WI) injury, which is manifested clinically as delayed graft function. The aim of this study was to define the effects of prolonged periods of WI injury on renal function in rat. Complete hilar clamping was performed by microvascular bulldog clamps and rat kidneys were subjected to 15, 60 and 90 minutes of in situ WI and then reperfusion for 1h. Blood were evaluated for Creatinine (Cr), blood urea nitrogen (BUN) and tumor necrosis factor-α (TNF-α) level. Reactive oxygen species (ROS), Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity was performed in ischemic tissue. The levels of BUN, Cr, ROS, TNF-α and kidney tissue MDA increased at a significant level, and SOD activity decreased at a significant level in the I/R group, compared with Sham Group ($p < 0.05$). Oxidative stress and inflammation parameters demonstrated the damage of kidney tissue subjected to prolonged WI. Altogether, these data show that prolonged WI period caused a severe decrement in renal function. The renal I/R model with 60 min of WI can be performed. This is a suitable translational model to study new early renal ischemic biomarkers and the pathophysiological mechanisms.

Keywords: *Kidney; Ischemia/Reperfusion Injury; Oxidative Stress*

Abbreviations

DCD: Donation after Circulatory Death; WI: Warm Ischemia; Cr: Creatinine; BUN: Blood Urea Nitrogen; TNF-α: Tumor Necrosis Factor- α; ROS: Reactive Oxygen Species; MDA: Malondialdehyde

Introduction

The Renal ischemia-reperfusion injury (IRI) causes renal functional alterations that might ultimately result in renal impairment [1]. Ischemia-reperfusion injury is an invariable consequence of many conditions including nephron sparing renal surgery, trauma and transplantation [2-5]. This organ dysfunction is caused by the effect of both ischemia and reperfusion on different renal cells. Ischemia results in damage of various parts of the cell whereas the return of blood flow to ischemic tissue can result in the recovery of normal function, paradoxically the tissue may also be injured during the process of reperfusion [2,5,6]. Despite decades of intensive research and an abundance of promising preclinical results, no interventions emerged that prevent or reduce clinical IR injury [4,7,8].

Research in the field of ischemia-reperfusion injury continues to be plagued by the inability to translate research findings to clinically useful therapies. This may in part relate to the complexity of disease processes that result in renal ischemia but may also result from inappropriate *in vivo* and *in vitro* research model selection [9,10]. Given the highly variable clinical presentation of diseases that involve ischemia, initial consideration of the animal model to select for study is of paramount importance. Animal models are indispensable for

unravelling the mechanisms of ischemia and reperfusion injury and for the development of new therapeutic strategies in its prevention or treatment [10,11], but each model has distinct advantages and disadvantages so that none of these models can perfectly recapitulate the natural onset and progression of human disease. Indeed, the development of novel therapeutic interventions against I/R-induced renal injury is a topic of intense research interest and depends in the choice of perfect animal model that mimic the clinical situation of injury during I/R.

Aim of the Study

The aim of this study is to provide and developed an animal models to be used for the study of ischemia-reperfusion injury, with the goal of allowing investigators to understand and select this models for development of novel therapeutic strategy based in biological compounds. As a result, this study also aimed to evaluate the model and several biochemical markers of renal oxidative damage and inflammation as potential predictors of IRI and have vital importance in evaluating the feasibility and efficacy of novel therapeutic interventions.

Materials and Methods

Animals

Male Wistar albino rats (200 - 250g) were housed in an air-conditioned room with a 12h light-dark cycle, with temperature (22 ± 2°C) and relative humidity (65 - 70%) kept constant. All experimental protocols were approved by the National Committee of Animal Care and Use. Rats were anaesthetized (60 mg/kg sodium pentobarbital, i.p.) during all surgical procedures.

Surgery and experimental protocol

To produce the renal I/R injury model in rats, anesthesia was induced by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Then the rat was placed on an operation plate equipped with a thermostat system. The abdominal cavity was opened along the ventral abdominal midline and the right renal artery, vein, and ureter were legated and ipsilateral nephrectomy was performed. The left renal pedicle was exposed and occluded with a non-traumatic vascular clamp for different time depending in animal groups. The renal pedicle was reopened later and reperfusion was performed. Rats were randomly assigned into 1 of 3 groups and either subjected to 15 min (Group 1, n = 8), 60 min (Group 2, n = 8) or 90 min (Group 3, n = 8) of warm ischemia (WI) prior to reperfusion for 1 h. Another group of rats underwent laparotomy only, where the kidneys were manipulated without nephrectomy or occlusion (sham-operated control group, n= 8). The blood sample was obtained after the end of the reperfusion period. None of the animals died during I/R period.

Renal Function Assessment

Blood samples from the aortic artery were collected at the time of sacrifice and immediately centrifuged, and the obtained serum was stored at -20°C until analyzed. The level of serum creatinine (Cr) and blood urea nitrogen (BUN) were detected using a standard clinical automatic biochemical analyzer (Hitachi7600-020).

Measurement of ROS Production

Lactate dehydrogenase (LDH) levels were measured as an indicator of cell injury in the tissue. The activity of LDH in the serum was quantified using LDH assay kit. This assay is based on the conversion of L-lactate and NAD to pyruvate and NADH by the released LDH.

Measurement of ROS Production

ROS were assayed according to the method of Bondy and Guo [12], using DCFH-DA which is deesterified in tissue homogenates to 2',7'-dichlorofluorescein acid and then oxidized by ROS to fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, to 10 µl of homogenate 990 µl of 0.1M phosphate buffer (pH 7.4) and 10 µl of 1.25 M DCFH-DA dissolved in ethanol were added. The reaction mixture was incubated at 37°C for 30 minutes, protecting the samples from light. The measurements were conducted using a fluorometer at wavelengths: Aex = 488 nm and Aem = 525 nm. ROS were evaluated using a standard curve for 10 µ MDCF, and were expressed in DCF µmoles per g tissue.

MDA content and SOD activity

The renal tissues, were collected at room temperature, homogenized, lysed and centrifuged at 12,000 x g for 10 minutes, following which the supernatants were collected. The levels of MDA and SOD in the renal tissues were measured using colorimetric assay kits according to the manufacturer's protocol.

Serum level of TNF-α assay

The serum level of TNF-α (tumor necrosis factor-α) was measured one hour after reperfusion using Quantikine TNF-α Rat ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The samples were tested in duplicate. Results were expressed as picograms of TNF-α per milliliter serum (pg/ml).

Statistical analysis

Statistical analysis was performed using Sigma STAT 3.5 (Jandel, USA). The paired Student's t-test was used when appropriate. Data are given as mean ± ESM. $P \leq 0.05$ was considered statistically significant.

Results

Serum level of BUN and Cr

Renal function was assessed by measurement of serum creatinine and blood urea nitrogen (BUN). The serum levels of Cr and BUN in I/R group were significantly higher than in Sham group ($p < 0.01$). However, in ischemic group of rats, an augmentation in the serum levels of BUN and creatinine was observed during reperfusion and this increase was modulated by the duration of WI in different groups (Figure 1 and 2).

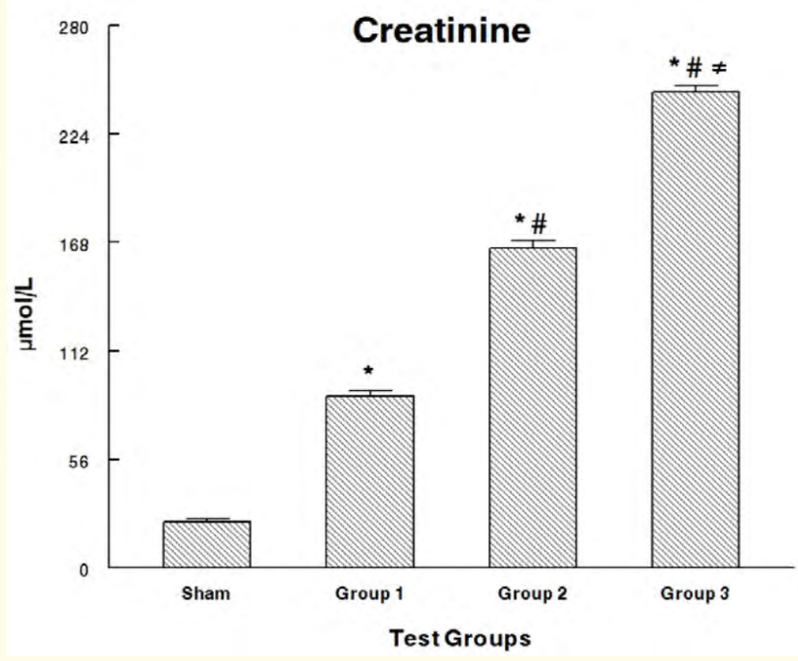

Figure 1: *Serum level of creatinine in different groups of rat. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minute of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1, ≠ P < 0.01, compared with the group 3.*

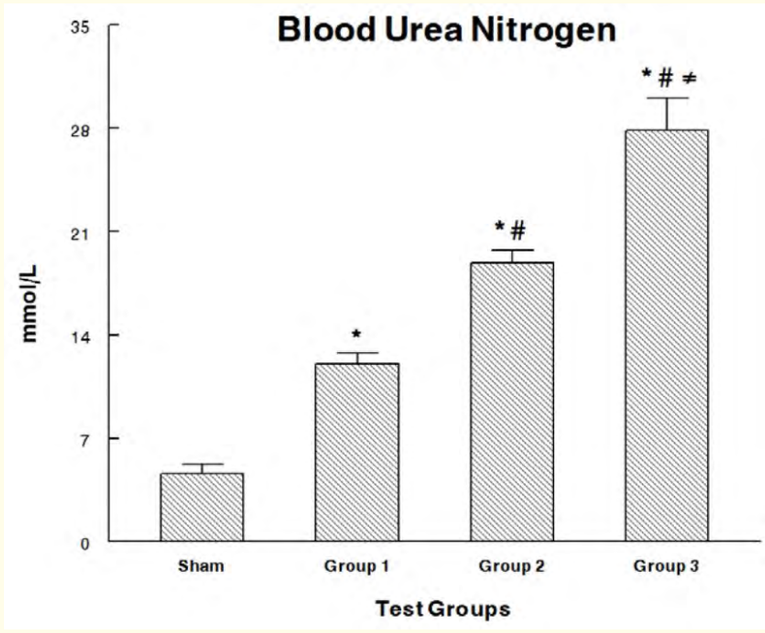

Figure 2: *Blood urea nitrogen. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minutes of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1, ≠ P < 0.01, compared with the group 3.*

Effects of ischemia time on TNF-α level in the rat kidney

TNF-α increased during reperfusion in the 15, 60 and 90 minutes WI groups. There was significant difference in the TNF-α level in different groups compared to sham group. The changes in levels were related to the duration of warm ischemia time (Figure 3).

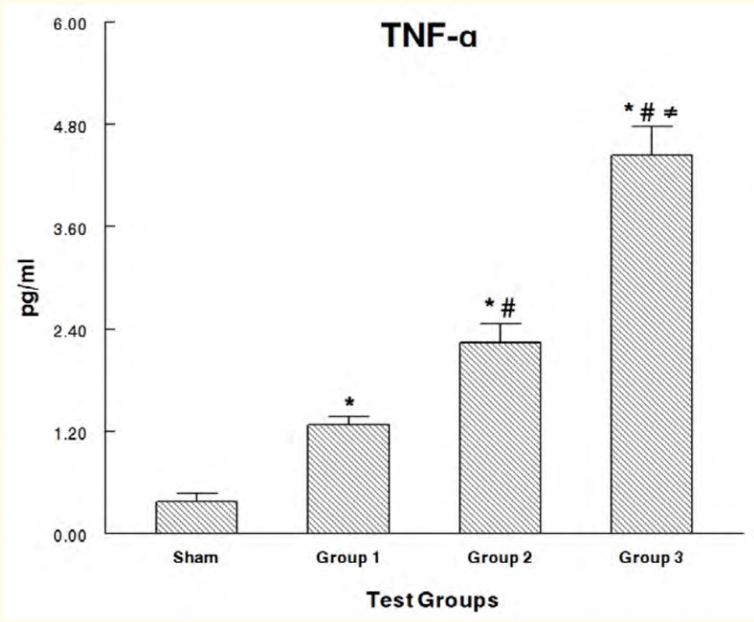

Figure 3: *Level of TNF-α. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minute of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1, ≠ P < 0.01, compared with the group 3.*

LDH activity

Kidney injury was assessed by the release of lactate dehydrogenase (LDH). LDH release increased during reperfusion in the 15 and 60 minutes WI groups but remained low and constant in the 90 minutes WI groups (Figure 4). Levels were significantly higher in the 15 minutes, 60 and 90 WI group compared to the sham groups at each of the hourly time points.

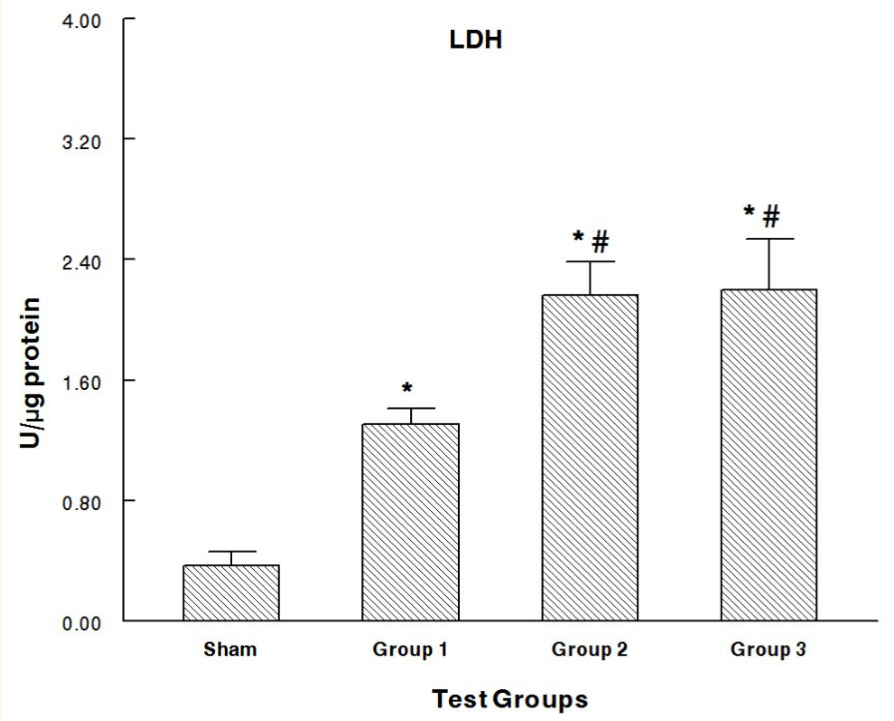

Figure 4: *LDH level in different rat groups. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minute of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1.*

ROS Production in I/R Kidney Injury

ROS production in kidney tissues was measured 1h after I/R in rats. After 1h of reperfusion significant increases in the tissue concentrations of ROS were observed in ischemic groups compared to sham group (Figure 5). This increase was modulated by the duration of WI in different groups.

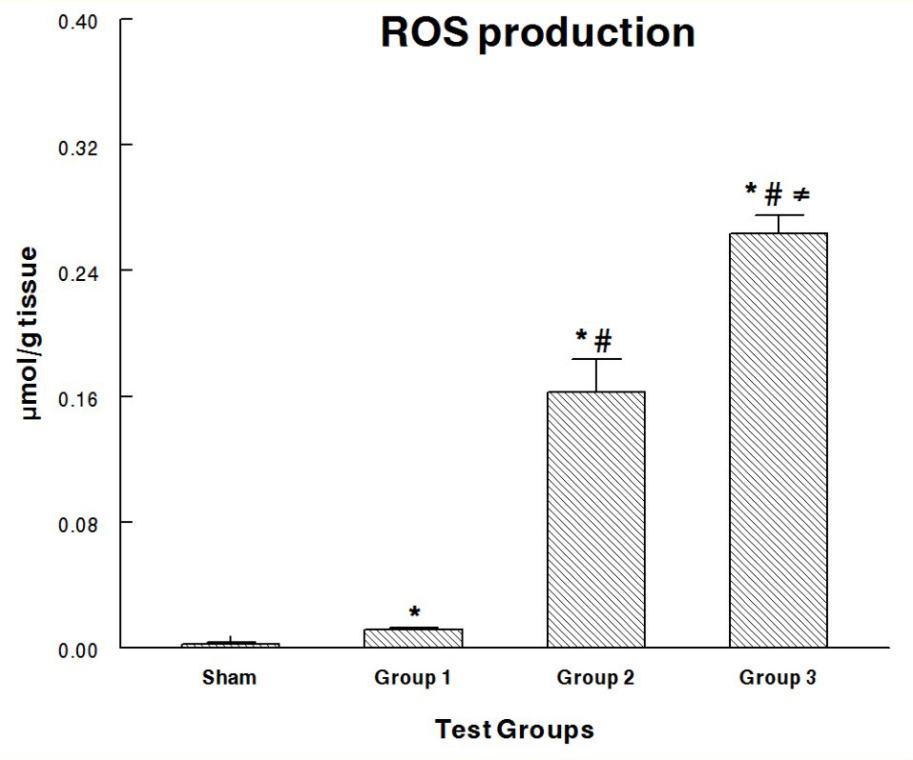

Figure 5: *ROS production in renal tissue of rats during reperfusion after different time of warm ischemia. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minute of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1, ≠ P < 0.01, compared with the group 3.*

Oxidative stress biomarkers in the kidney

The level of MDA in renal tissues was induced by I/R and was significantly higher than that in the Sham group ($p < 0.01$). The level of MDA in renal tissues reached 1.4 ± 0.2 nmol in the Sham group, whereas the level of MDA in the renal tissues of I/R group 3 reached 7.5 ± 0.1 nmol (Figure 6). On the other hand, the SOD activity in renal tissues was significantly less in I/R group than in the Sham group ($p < 0.01$). The activity of SOD in renal tissues was affected by the time of WI in I/R group during reperfusion (Figure 6).

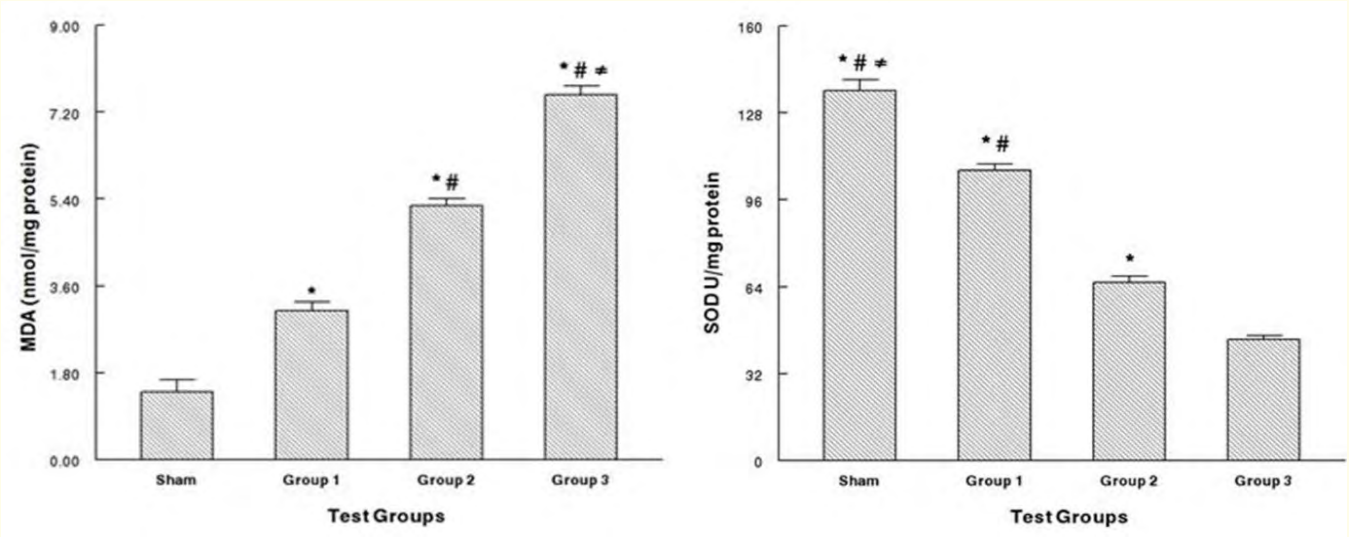

Figure 6: *The levels of lipid peroxidation products (MDA) and SOD activity in the rat kidney. Sham: kidneys were manipulated without warm ischemia (sham-operated control group); Group 1: 15 minute of warm ischemia and 1h of reperfusion; Group 2: 60 minute of warm ischemia and 1h of reperfusion; Group 3: 90 minute of warm ischemia and 1h of reperfusion. Results are expressed as mean ± SEM, n = 8 rats per group. *P < 0.001 compared with the sham group; # P < 0.001 compared with the group 1, ≠ P < 0.01, compared with the group 3.*

Discussion

As a common renal disease in clinical practice, acute renal injury is caused by renal ischemia-reperfusion. Ischemia/reperfusion (I/R) is the determinant factor for the early functional recovery of renal transplantation [13,14].

Many therapy processes has emerged as a potential treatment for acute renal injury affecting transplanted kidneys. Unfortunately no treatments are in widespread clinical use [15-17]. Although the changing trends in organ donation and to meet the demand of organs have led clinicians to utilize organs from older and less fit deceased donors [18,19]. Such kidneys remains limited as organs are exposed to a significant period of warm ischemia (WI) prior to retrieval and have poorer patient outcomes [20,21]. The absence of interventions for the treatment of renal IRI reflects the lack of relevant animal models in which novel therapies may be easily studied. Commonly used rodent models of renal IRI lack the capacity to inflict a severe, long-standing renal injury without excessive post-operative animal deaths [15]. To address this, we investigated the use of prolonged warm ischemic times in order to create a severe and sustained renal injury model. In the current study, WI induced renal damage evidenced by serum biochemical and tissue parameter changes as well as histopathological damage during reperfusion. BUN and serum creatinine were elevated indicating renal impairment. Similarly, LDH was found to be significantly elevated indicating nonspecific cellular injury. We identified that progressively longer warm ischemia times were associated with an increased injury.

Although warm ischemia is well known to cause acute kidney injury after partial nephrectomy in non-transplant patients [22,23], there have been fewer clinical studies examining its impact on kidney transplant function and patient outcomes after transplantation. Therefore, it is biologically plausible that even small changes in warm ischemia time influence long-term outcomes [24]. This study shows that Rat kidneys subjected to prolonged warm ischemic injury of 15 - 90 minutes have very poor early renal function after reperfusion. There was an increase in serum creatinine and blood urea nitrogen that correlate with the duration of ischemia.

As expected, 15 minutes of WI had very little adverse effect. Fifteen minutes was chosen as the minimal time under study as a reflection of the usual mean warm ischemic time in human kidneys transplanted from controlled donation after circulatory death DCD [25]. These kidneys had high blood flow, produced a large volume of urine and demonstrated good renal function. Kidneys in the 60 min WI group (group 2) were not able to maintain a low level of creatinine and BUN over the 1h of reperfusion period. Parekh., et al. concluded that kidneys can safely tolerate 30 - 60 minutes of ischemic injury and this is consistent with the findings described here using the rat model [26]. This duration of warm ischemia did not affect tissue viability. In order to create a severe and sustained renal injury model, Wang., *et al.* investigated the use of prolonged warm ischemic times [27]. They observed that warm ischemic times of up to 90 minutes was associated with long-term disruption of renal architecture, increased levels of apoptosis and renal fibrosis and resulted in almost 80% animal deaths at 4 weeks.

Oxidative stress constitutes the mechanism of production and progression of numerous renal diseases. In the present study I/R injury causes an increase in the level of ROS and lipid peroxidation products, and reduction of anti-oxidant defense systems. The elevated levels of lipid peroxides resulting from increased formation of free radicals are recognized as one of the possible biochemical mechanisms for I/R-induced tissue damage. This is in agreement with previous studies where oxidants have been shown to be elevated due to I/R [10,28,29].

This study of I/R aims to provide a well-characterized animal model with strong acute responses to ischemic insult similar to the human response and not to mimic a specific disease, but rather, provide common pathophysiological changes observed in various kidney diseases and during transplantation. The model of renal IRI detailed here uses a midline laparotomy approach to induce ischemia in the left kidney using clamps and a right nephrectomy. As illustrated by the representative results, modifying the duration of ischemia can control the severity of injury. Therefore this model can be adjusted to induce mild, moderate or a high level of kidney injury as required by the experimental question posed.

Conclusion

In conclusion, this study demonstrated a clear association between increasing warm ischemic time and both greater severity of IRI and deterioration in renal function. We suggested that renal artery clamping for 60 minutes is more appropriate during renal warm ischemia/reperfusion. Hence, with this model we are able to screen potential therapies' efficacy prior to their use in a more technically complex transplant model and to test potential drugs for clinical transplantation.

Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Volume 5 Issue 7 July 2018