Animal model of chronic kidney disease using a unilateral technique of renal ischemia and reperfusion in White New Zealand rabbits[1]

Eliane Barbosa Togoe[I], Iandara Schettert Silva[II], Juliana Loprete Cury[III], Albert Schiaveto Souza[IV], José Henrique Saraiva Borges[V], Klaus Casaro Saturnino[VI]

[I]Master, Health and Development Postgraduate Program, Federal University of Mato Grosso do Sul (UFMS), Campo Grande-MS, Brazil. Conception and design of the study, acquisition and interpretation of data, manuscript preparation.
[II]PhD, Postgraduate Program in Health and Development, UFMS, Campo Grande-MS, Brazil. Guidance and experimental design of the study, veterinary conductor of the experimental surgery.
[III]Master, Fellow PhD degree, Postgraduate Program in Health and Development, UFMS, Campo Grande-MS, Brazil. Associate Professor, Physiotherapy Department, University Center of Grande Dourados (UNIGRAN), Dourados-MS, Brazil. Design of the study, interpretation of data, critical revision.
[IV]PhD, Associate Professor, Department of Morphophysiology, UFMS, Campo Grande-MS, Brazil. Statistical analysis.
[V]PhD, Associate Professor, Veterinary Hospital Unit, UNIGRAN, Dourados-MS, Brazil. Veterinary assistance in the experimental study.
[VI]Master, Associate Professor, Department of Veterinary, UNIGRAN, Dourados-MS, Brazil. Histopathological examinations.

ABSTRACT

PURPOSE: To establish a model of chronic kidney disease in White New Zealand rabbits, using an exclusive unilateral technique of renal ischemia and reperfusion.

METHODS: Twenty males White New Zealand rabbits were used. All animals were subjected to the following: pre-surgical blood collection (1st collection) for creatinine and urea serum analysis, left renal ischemia and reperfusion surgery technique, another blood sample was collected after 6 weeks post surgery (2nd collection), the last blood sample (3rd collection) blood sample was taken 11 weeks post surgery (pre-euthanasia), euthanasia and withdrawal of right and left kidney for histopathological analysis.

RESULTS: The creatinine levels after surgery was statistically significant higher in the 3rd collection, regarding the 1st and 2nd collection (p<0.05). A significant statistic increase for urea was showed only in the 2nd collection (p<0.05) when compared to the 1st and 3rd collections. Histopathological analysis showed bilateral lesions in the renal tissue, consistent to the process of ischemia and reperfusion.

CONCLUSION: This exclusive unilateral technique of renal ischemia and reperfusion without nephrectomy in White New Zealand rabbits, showed effectiveness in getting an animal model of chronic kidney disease

Key words: Renal Insufficiency, Chronic. Models, Animal. Rabbits.

Introduction

Chronic Kidney Disease (CKD) is considered one of the major health problems in the world and a significant cause of morbidity and mortality. According to the last two Brazilian Dialysis Census, published in 2012 and 2014, the number of people on dialysis in Brazil has progressively increasing over the years, with an incidence rate of 149 patients per million person[1,2]. CKD is a worldwide epidemy that can be explained by the significant growth of the diabetes mellitus prevalence, cardiovascular disease numbers and the increase of worldwide life expectancy[3].

The course of CKD is characterized by irreversible, progressive and slow loss of the renal function, which affects diverse systems of the organism, becoming a pathology of difficult approach since the CKD patient may present complications in multiple organs and functions[4]. Thus, the CKD approach requires a wide variety of interventions, which can be difficult to study in human design methodology.

In view of this problem, animal models of CKD by surgical intervention to reproduce its clinical and pathology has been developed, and thus, therapeutic methods tested. There are some protocols in the literature for the development of an animal model of CKD in rats. A two time surgery in which the animal is first subjected to performing ischemia and reperfusion surgery, and after a period ranging from four to 12 weeks, the animal undergo a new surgical procedure to accomplish reduction of renal mass by nephrectomy at different rates 2/3, 4/5, 5/6[5,6]. This type of surgical technique results in a reduction of animal´s survival and in association with a low volemia of rats jeopardizes the use of this animal model on certain types of experiments, especially methodologic designs requiring long periods of observation. Considering these difficulties, regarding the volemia and survival after experimental induction, it has been proposed, the development of animal models of renal failure in other animal species that can respond more adequately to all necessary procedures.

The White New Zealand rabbit is an alternative in animal models of CKD, as it has the best prospect of drafting technique and survival after CKD induction, besides it offers a therapeutic window of 12 weeks and greater blood volemia compared to rats, which favors the consecutive blood samples for biochemical analysis[7].

Renal ischemia is the major cause of CKD, triggered by the loss of oxygen supply, nutrients and the removal of wasting products from the renal cells. The tubular epithelium plays a main role in the development of renal deterioration after ischemic injury, especially through cytokines production[8,9]. There are a lot of states that may generate physiopathological renal ischemia / reperfusion, among them, Hypertension and Diabetes Mellitus, which are the two leading causes of CKD in humans and increasingly prevalent among Brazilian people[10,11].

Thus, the aim of this study was to establish a model of CKD in White New Zealand rabbits, using an exclusive unilateral technique of renal ischemia and reperfusion.

Methods

The study was approved by the Animal Use Committee of Mato Grosso do Sul Federal University (UFMS), under # 280 protocols. Twenty male White New Zealand rabbits, weighing 2.5 to 3 kg, sourced from Maringa State University (UEM) were used. The animals were kept in individual cages in a room with constant ventilation and appropriate temperature, with free access to water and commercial feed.

All animals were subjected to the following procedures: first pre-surgery blood sample collection, surgery to access the left kidney and ischemia/reperfusion process, second blood collection after 6 weeks post surgery, third blood sample after 11 weeks of post-surgery (pre-euthanasia), euthanasia. The blood samples were used for creatinine and urea serum measurements.

The first blood sample was taken immediately before surgery, after the administration of the following preanesthetic medications: intramuscular injection with acepromazine (0.05g/kg) associated with butorphanol (1mg/Kg), 15 minutes before anesthesia. Blood samples were obtained by cardiac puncture, performed by the veterinarian in charge of the research. To the blood sample collection, the following materials were used: disposable syringe, needle 25/7, descarpak, anticoagulant tube for blood collection, cotton and alcohol 70% and procedure gloves.

For anesthesia, the following medications were used: dissociative anesthetic (tiletamine hydrochloride and zolazepam hydrochloride) at a dose of 5mg/kg by intramuscular injection. To access the left kidney, a left laparotomy was performed by a longitudinal incision of 5 cm. After location of the renal hilum, clamping of the vascular pedicle on the left kidney was held during twenty minutes to generate renal ischemia. After this period the clamps were removed, reperfusion of the kidney and subsequent plans synthesis with a 4-0 mononylon. The entire surgical procedure was performed by the veterinarian responsible for the research and co-workers in the Veterinary Surgical Center at the Veterinary Hospital of the University of Grande Dourados (UNIGRAN).

The animals were kept under observation for six weeks after surgery, receiving daily hygiene care, free access to water and commercial feed. At the end of this step, the second blood collection was performed the same way of the first one.

After more five weeks observation, the third and last blood pre-euthanasia collection was taken. The euthanasia was performed by intravenous barbiturate overdose (100mg/kg), followed the withdrawal of the right and left kidneys for histopathology.

The first collection represents normal values for the studied sample, the second and third ones represent the variation of these parameters as a function of time after renal and ischemia/reperfusion surgical procedure in the left kidney.

After the histopathological material collection, the pieces were immersed in 10% formaldehyde and processed for paraffin blocking (5 cm). Then the pieces cut on microtome at 5 micrometers thickness and stained with hematoxylin and eosin (HE).

A pathologist evaluated the slides using Nikon Eclipse E200 electron microscope and the blades analysis used to establish the presence or absence of renal lesions in both kidneys.

To summarize the data of quantitative variables of urea and creatinine, the mean value and standard deviation were used. For statistical comparison of the three time-collection of these variables were used the One-way ANOVA for repeated measure with post hoc Tukey test, considering significance statistic set $\alpha=5\%$ ($p<0.05$). Qualitative variables concerning the histopathological analysis were summarized in absolute and relative frequency (%).

Results

A total of twenty rabbits underwent the ischemia and reperfusion surgeries, three of them died throughout the research and were excluded from the final analysis.

The animal model of CKD in White New Zealand rabbits showed good survival and allowed consecutive blood collections without compromising the blood volemia of the animal. In Figure 1, the change in color of the left kidney after 20 minutes of ischemia during the surgical procedure was observed.

FIGURE 1 – Picture showing the change in coloration of the left kidney after 20 minutes of ischemia and reperfusion procedure.

The results for serum creatinine levels showed a gradual increase after surgery, and the values of the 3rd collection were higher and statistically significant ($p<0.05$) compared to the 1st and 2nd collection (Table 1). This value demonstrates a decline in glomerular filtration rate over time.

TABLE 1 – Table showing the mean value and standard deviation of creatinine and urea levels in the three collections.

	1st Collection	2nd Collection	3rd Collection
Creatinine	1.01±0.16	1.15±0.16	1.73±0.95*
Urea	31.0±4.53	39.61±3.63#	32.62±5.79

Creatinine and urea values presented in mg/dl
*Creatine values statically significant ($p<0.05$) in comparison to the 1st and 2nd collection
#Urea values statically significant ($p<0.05$) in comparison to the 1st and 3rd collection

Urea levels showed a different behavior, there was a significant statistical increase ($p<0.05$) in the 2nd collection, demonstrating a change in renal filtration of this metabolic (Table 1). However, the 3rd collection urea levels showed a decline, reaching almost the 1st collection values.

Table 2 shows the lesions found in both kidneys of the 17 animals that were included in final analysis, summarized in values of percentage and absolute number of occurrence in animals.

TABLE 2 – Table shows the lesions found in both kidneys, summarized in values of percentage (absolute number) of occurrence in animals.

Histopathologycal Changes	Right Kidney % (n)	Left Kidney % (n)
Cortical and Medullar Congestion	70.5 (12)	35.2 (6)
Lymphocytic Infiltrate	17.6 (3)	11.7 (2)
Hydropic Degeneration Tubular Cells	5.8 (1)	33.2 (6)
Bowman Area Increase	0	11.7 (2)
Glomerular Retraction	0	11.7 (2)
Repletion of Glomerular Capillaries	5.8 (1)	11.7 (2)
Glomerulonephritis	11.7(2)	11.7 (2)
Glomerular atrophy	0	35.2 (6)
Fibrosis	0	11.7 (2)

Histopathologycal changes in both kidneys: right and left. Total n=17 animals.

The histopathological analyzes showed that the injuries occurred in the renal tissue of the left kidney, which underwent the process of ischemia and reperfusion, and the right kidney, which did not undergo any intervention. These results observed in the righ kidney, are consistent with the process of reperfusion after ischemic induction since mediators that induce lesions in other tissues and organs not subjected to the ischemia are produced.

The figures below show some of the histopathological lesions observed in the kidney tissue slides. In Figure 2 we can observe cortical and medullar congestion in the right and left kidney, in Figure 3 we can observe glomerular atrophy and hydropic degeneration in the left kidney and Figure 4 shows glomerulonephritis in the right kidney.
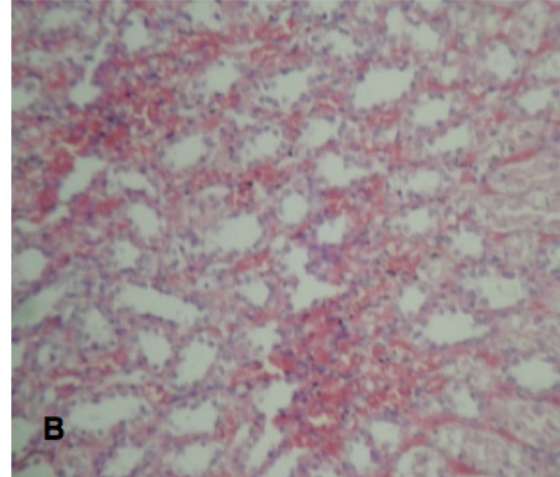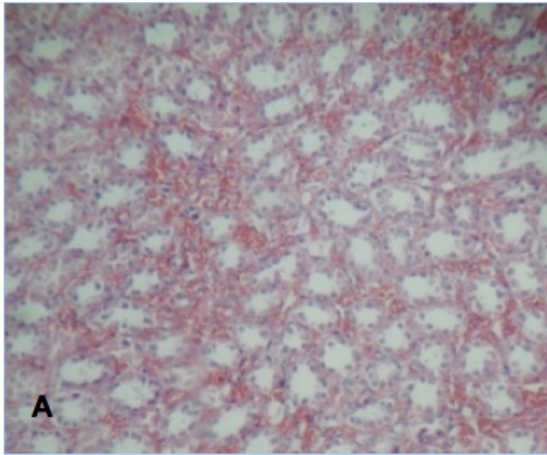

FIGURE 2 – Hystophatological slide photograph of left kidney (**A**) and right kidney (**B**) demonstrating cortical and medullar congestion (increase x10 HE).
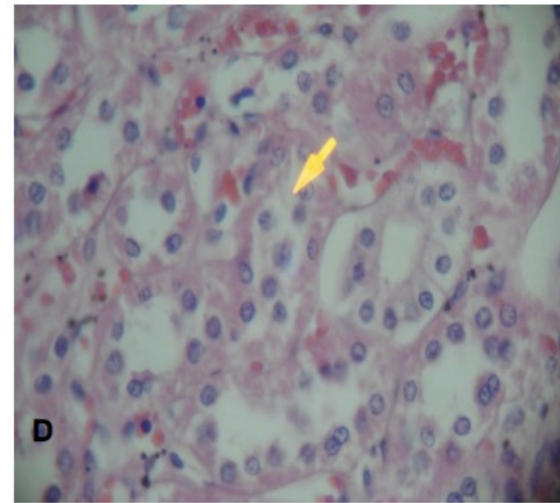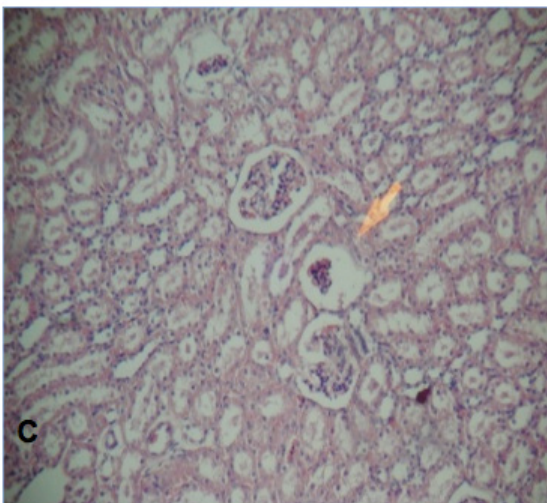

FIGURE 3 – Hystophatological slide photograph of left kidney showing glomerular atrophy (**C**) and hydropic degeneration of tubular cell (**D**) (increase x40 HE).
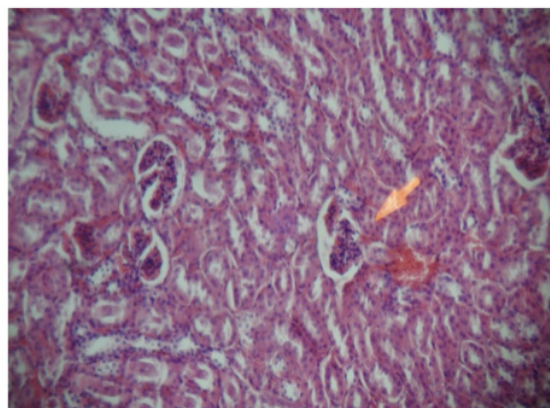

FIGURE 4 – Hystological slide photograph of the right kidneydemonstrating glomerulonephritis (increase x10 HE).

Discussion

It was observed through this study that the unilateral technique of renal ischemia and reperfusion is feasible, and produces renal lesions compatible with CKD in White New Zealand rabbits.

Some studies conducted to establish an animal model of chronic kidney disease, used the two-surgical-times procedure, i.e., a first surgery is performed to establish the process of renal ischemia by clamping the unilateral renal pedicle and after a period that varied from four to 12 weeks, according to each protocol, a new surgical procedure is performed for the reduction of the renal mass to the contralateral organ that suffered the ischemic procedure[5-7].

Santos *et al.*[5] examined the biochemical and morphological effects on the 60 remnant kidney´s rats subjected to progressive ablation surgery of the renal mass equivalent to ½, 2/3 e 5/6, after eight weeks the animals were re-operated to remove the remaining kidney. This animal model showed considerable changes in the creatinine level, proteinuria and hypertrophy of the kidney, although the authors did not report the difficulties of this kind of animal model as well as the number of deaths.

Costa *et al.*[7] established a model of chronic renal failure in White New Zealand rabbits, using bipolar left nephrectomy and after four weeks, a right nephrectomy. The authors observed an increase progress in urea, creatinine, sodium and left kidney hypertrophy.

Knopka *et al.*[6] evaluated a model of chronic renal ischemia in Wistar rats. The animals underwent a partial ligation of the left renal artery and after seven days a left nephrectomy. The authors observed a progressive reduction on weight, volume, renal cortical thickness, proximal tubular atrophy and infiltrate. The biochemical changes were not evaluated, however, according to the authors, the study provided a structural and morphological analysis of the animal model.

The available techniques in the literature induce changes consistent to the clinical course of CKD (increasing urea and creatinine levels, proteinuria and histopathological renal changes). However, they are difficult to be developed, since it involves two-time-surgery, which can lead to a reduction of the animal survival. Moreover, the choice of animal model, the Wistar rats, due to their limited size and low blood volemia, impair blood samples for biochemical analyzes and the reduction of animal survival in the post-surgery[5-7]. Thus, in this experiment we chose to use the White New Zealand rabbits and one surgical time procedure, through the exclusive unilateral technique of renal ischemia and reperfusion.

The choice of the White New Zealand Rabbit for this experiment was due to their better survival after CKD induction, with the possibility of providing a therapeutic window of 12 weeks agreeing with Costa *et al.*[7]. When considering the life expectancy of a rabbit, around seven to eight years, approximately ten times smaller than human beings, we can assume that the observation interval of 12 weeks is equivalent to two and a half years on *anima móbile*, which features enough time for CKD installation. Moreover, they present a higher volemia, compared to the rats, facilitating the samples collections for biochemical analysis. In the present experiment, consecutive blood samples for biochemical analysis were taken, without compromising the lives of animals that remained viable around 11 weeks until euthanasia.

The exclusive unilateral technique of renal ischemia and reperfusion led to a continuous increasing in creatinine levels after the CKD induction. Renal clearance of creatinine has a long history as a marker of GFR. Spanaus *et al.*[12] presented a study that strengthens the use of creatinine as a reliable marker for CKD. These authors compared the performance of two low molecular weight markers, cystatin C and β-trace protein (BTP), with creatinine serum for the diagnosis, staging and prediction of progression in a cohort of 227 patients with primary non-diabetic CKD and concluded that the three biomarkers are equivalent in terms of diagnostic performance - even in discrete degrees of deterioration of renal function - and in terms of predicting risk for progression. These authors stated that any serum biomarker of GFR should obey the laws of physics: with declining GFR, serum concentration should increase. The fact of the relationship between serum concentration and GFR be a reciprocal function, explains how relatively small changes in concentration, that occur in the early stages of decline in kidney function are followed by accelerated increases. Thus, when analyzing the data obtained in blood samples in this study after 11 weeks of renal ischemia and reperfusion procedures, serum concentrations increased consistent to the decreased of glomerular filtration.

During the surgical procedure, to generate ischemia and reperfusion, the left renal pedicle was clamped for 20 minutes. It was believed that in comparison to the heart and brain, the kidneys could recover completely from an ischemic intervention. However, this has been occurring with less frequency, recognizing that ischemia/reperfusion (more than 20 minutes) can lead to CKD[13]. Thompson *et al.*[14] studying the risk related to the time of renal ischemia in clinical impact, suggests that the vascular clamping of the renal pedicle is associated with the increased risk of chronic deterioration of renal function. His study demonstrated that ischemia for more than 20 minutes is associated with the development of CKD[14]. In our experiment a 20 minute-renal ischemia was sufficient to generate lesions proved by histopathological analyzes.

Renal ischemia/reperfusion injury is the result of a deficiency of oxygen and nutrient delivery to the removal of wasting through tubular cells. There is a mismatch between the demand and supply of O_2, with the accumulation of substances, metabolism products. As a result, of an imbalance, cells of the renal tubular epithelium suffer severe damage, such as cell death by apoptosis and necrosis, creating disturbances in the water and electrolyte homeostasis and consequent reduction in the excretion of waste products from metabolism[8].

One reason for the renal sensitivity ischemia is that the renal microvasculature is highly complex, and must meet a high demand for energy. This delicate balance between supply and demand for O_2 is easily disturbed in cases of ischemia[15]. A study has shown that during ischemia/reperfusion blood flow to the renal medulla is reduced disproportionally to the reduction of renal perfusion. The vascular endothelium is injured and small renal arterioles undergo vasoconstriction in response to high tissue levels of chemical mediators of inflammation (endothelin-1, angiotensin II, thromboxane A2, and leukotriene) and stimulation of the peripheral nervous system. In addition, there is a decrease of the vasodilatation of arterioles due the decline of nitric oxide and other vasodilators, associated to the increase of vasoactive cytokines (TNF-α, IL, endothelin), generating as a result, activation and adhesion of leukocytes to the endothelium. The edema formation further contributes to arteriolar vasoconstriction, resulting in interference on the blood flow to the proximal tubule and loop of henle, which are usually hypoxic due to exchange properties with straight renal vessels[8].

Clinically all these changes reflect an increased injury during the reperfusion phase, when the O_2 consumption increases with the demand of tubular transport. Thus, the initial injury leads to a cascade of events that culminate in vascular injury, vascular congestion and reduced ability to neutralize the toxic radicals, a fact that undermines the renal filtration and leads to a progressive increasing levels of urea and creatinine[16]. Corroborating the literature, this experiment demonstrated the loss of renal function through the progressive increase in creatinine levels, caused by unilateral ischemic process.

The consequence of an ischemic process of renal injury is an important cause of CKD in humans. In this sense, Arterial Hypertension (AH) plays an important role in renal ischemia because both accelerated and chronic forms may provide a framework for kidney injury of microvascular nature, characterized by myointimal proliferation and fibroid necrosis. Hyaline arteriosclerosis that installs due to AH generates the process with consequent ischemic injury of renal microvasculature, featuring hypertensive nephrosclerosis. Considering the high prevalence of hypertension in the population, a significant number of patients with renal dysfunction can be predicted and identified in our environment as the second leading cause, after diabetic nephropathy in patients starting dialysis each year, according to data of the Health Department of Brazil[17,18].

This exclusive unilateral technique of renal ischemia and reperfusion is quite useful for the CKD induction process, as renal ischemia is related to diseases so prevalent in the community, such as Arterial hypertension and Diabetes Mellitus[19].

Conclusion

This experiment using an exclusive unilateral technique of renal ischemia and reperfusion, without a second-time surgery for nephrectomy, in White New Zealand rabbits, showed effectiveness in getting an animal model of Chronic Kidney Disease.

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Correspondence:
Eliane Barbosa Togoe
Rua Ciro Melo, 3273
79805-031 Dourados – MS Brasil
elianetogoe@hotmail.com