ISCHEMIC AND REPERFUSION SYNDROME OF HIND LIMBS: FUNCTIONAL AND HISTOLOGICAL RENAL CHANGES IN RATS*

SÍNDROME DE ISQUEMIA E REPERFUSÃO DOS MEMBROS POSTERIORES: ESTUDO FUNCIONAL E HISTOLÓGICO RENAL EM RATOS

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TAKITO AM, SILVA JCCB , BUENO V, FRANCO M, BURIHAN E. Ischemic and reperfusion syndrome of hind limbs: functional and histological renal changes in rats. Medicina (Ribeirão Preto) 2005, 38 (3/4): 294-300.

Abstract: Ischemic and reperfusion injury of the extremities may result in a systemic, severe and complex metabolic syndrome, manifested by acute renal failure, myoglobinuria, metabolic acidosis, hipercalemia and free radicals releasing. We investigated the functional and histologic renal changes after ischemia and reperfusion of the hind limb skeletal muscles. Rats were submitted to the ligature of the infrarenal aorta for 6 and 12 h. The animals were then randomized into four groups of 10 rats: Group I, 6 h of ischemia and 24 h of reperfusion; Group II, 12 h of ischemia and 24 h of reperfusion; Group III, 6 h of ischemia and 10 days of reperfusion; and Group IV, Group *sham* with no ischemia or reperfusion. Blood samples at the end of the experiment and urine volume in the first 24 h of reperfusion in group I and II and in the last day in group III for functional analysis were collected. The folowing renal functional parameters were studied: . creatinine plasmatic level, creatinine depuration and sodium urinary/creatinine urinary ratio. The kidneys were removed and a histological tubulo-interstitial lesional index was evaluated for each animal. We found higher plasma creatinine levels and morphologic changes in groups submitted to ischemia and reperfusion. Ten days after reperfusion, the histologic changes persisted despite the recovery of renal function.

Keywords: Ischemia. Reperfusion. Kidney.

1- INTRODUCTION

The reestablishment of the arterial flow to an extremity following acute arterial occlusion usually results in morphologic and functional recovery. However, in a small number of instances, even if arterial patency is achieved, a systemic, complex, metabolic syndrome may develop leading to the loss of the limb and even death[1]. The sudden restoration of blood flow to a previously ischemic extremity results in a mas-

Research supported by FAPESP 97/06326-0

sive wash out of lactate, potassium, myoglobin, with resultant systemic acidosis, hypercalemia, mioglobinuria and free radicals releasing. Simultaneously, the acutely revascularized limb develops massive edema, hemoconcentration and hypovolemia with subsequent impairment of the renal function[2/5]. The pathogenesis of acute renal failure following rhabdomyolysis has been attributed to several mechanisms: **i**) Myoglobin nephrotoxicity impair renal function, mainly when dehydratation, acidemia, or both coexists[6,7,8]; **ii**) Primary reduction of glomerular filtration rate due to cortical and glomerular hemodynamic changes due to hipotension after restoration of the blood flow to the extremity[9]; **iii**) Myoglobin cast producing tubular obstruction and tubular acute necrosis[10,11,12]; **iv**) Release of oxygen-derived free radicals mediating back leakage of filtrate through damaged tubular renal epithelium, with loss of renal excretory function[13,14,15].

The renal tubule performs functions critical to internal homeostasis, including reabsorption of filtered solutes and water, secretion of metabolic products, maintenance of acid-base balance and regulation of fluid volume. Injury of this epithelium may result in profound effects on those critical functions[16]. After acute renal tubular injury, there occurs a regenerative repair phase which results in a gradual return of structural and functional integrity over a period of a few days up to several weeks or even one year[17]. The aim of the present study was to evaluate the functional and morphological effects of ischemic and reperfusion skeletal muscle injury in the kidneys.

2- MATERIAL AND METHODS

The experimental model consisted in an ischemic-reperfusion skeletal muscle injury in the hind limbs of adult Wistar EPM-1 male rats from the Central Animal House of UNIFESP, aged 90 to 120 days and weighing 250 to 350 g. Ethical principles for animal experimentation as stated by the International Animal Protection Union and Law 6638 of May 1979, and revised in 1983 were strictly followed. The protocol was submitted to the Research Ethics Committee of UNIFESP and approved. Forty animals received an anesthetic dose of ethyl ether. A random selection was performed by sealed envelopes to divide the animals into four groups of 10 rats each. After abdominal trichotomy and antisepsis using topical iodopovidine, a median 5 cm laparotomy was carried out, moving the bowel to the right. The juxta-infrarenal portion of the abdominal aorta was identified. Ligature efficacy was confirmed by the paleness of the hind limbs and the absence of a pulse below the ligature. Next, the intestines were repositioned in the cavity and the abdominal wall was closed in a continuous single-plane suture. Reperfusion was achieved by removing the ligature of the abdominal aorta; the restoration of the blood flow to the extremities was confirmed by the recovery of perfusion of the hind limbs and the presence of a pulse below the ligature with a microscopic view. Four groups of animals were studied (n=40):

Group I (n=10) - Rats were submitted to 6 h of ischemia and 24 h of reperfusion. Six hours later the animals were reoperated on and the ligature of the animal's aorta was undone. The urinary volume was collected during the 24 h of the reperfusion. After 24 h of reperfusion, the animals were anesthetized with of ethyl ether and 3 ml of blood was collected from the aorta and the animals were killed by exsanguination. The kidneys were then removed to histological analysis.

Group II (n=10) - Rats were submitted to 12 h of ischemia and 24 h of reperfusion. Twelve hours later the animals were reoperated on to allow reperfusion of the hind limbs. The urinary volume was collected to analysis during the 24 h of reperfusion. After 24 h of reperfusion, the animals were anesthetized and blood was collected from the aorta, and the animals were killed by exsanguination. Kidneys were removed to analysis.

Group III (n=10) - Rats were submitted to 6 h of ischemia and 10 days of reperfusion. Six hours later the animals were submitted to a new surgery and the ligature of the abdominal aorta was undone. During the last 24 h of reperfusion the urinary volume was collected. From this point on, the procedure was again identical to that of group I and II.

Group IV (n=10) - *Sham* animals where no ischemia or reperfusion, were anesthetized, submitted to a median 5 cm laparotomy, with the isolation of the infrarenal portion of the abdominal aorta which was not ligated. The abdominal wall was closed. The urinary volume was collected during 24 h. After 24 h of laparotomy, the animals were anesthetized and blood was collected. From this point on, the procedure was identical to that of group I; II and III.

All laboratory testing were measured using automatic analyzers. Functional renal analysis was performed by the plasmatic creatinine level; creatinine clearance and sodium urinary/creatinine urinary ratio. Kidneys were split longitudinally and fixed in 10% for-

malin for histological analysis. 4 μm thin sections from paraffin blocks were stained with hematoxylin-eosin for structural optical microscopic analysis. The tubulo-interstitial changes were histologically evaluated semi-quantitatively in a 0-3+ scale. The parameters studied were: edema, capillary congestion and hemorrhage for the interstitial compartment; hydropic and hyaline degeneration, cellular descamation, cellular necrosis for the tubular compartment. Therefore the highest lesional index was 9 for the interstitium and 15 for the tubules.

The Kruskal-Wallis nonparametric analysis of variance test to detect statistical differences among groups I, II and IV, and the Mann-Whitney test for statistical comparison between groups I and III were used[18,19]. For all comparisons, differences were considered significant for $p \leq 0.05$ with a 95% confidence interval.

3- RESULTS

Animals from groups I and II showed significantly increased plasmatic creatinine levels when compared to those from group IV ($p < 0.05$) (Table I). In ischemic-reperfusion groups, the 24h values of creatinine clearance were lower than controls, although this difference was not statistically significant (Table I). The skeletal muscle injury in groups of 6 and 12 h of ischemia developed statiscally significant alterations in the renal tubular and interstitial compartments compared to the control group ($p<0.05$; Table II). The functional renal evaluation showed that the values of plasmatic creatinine levels and Na/creatinine urinary ratio were lower in group III (10 days of reperfusion) compared with group I (24 h of reperfusion) in ischemic skeletal muscle injury of 6h (Table III). Although the histological renal lesional indices were significantly higher in groups I and III as compared to control group, there was no difference statistically significant when we compared I and III (Table II), (Fig. 1 and 2).These results demonstrated that in 10 days of reperfusion, although there were still histological lesions in renal tubules, the renal function was already recovered.

Table I - Plasmatic creatinine (mg/dl) and creatinine clearance (ml/min) after 24 h of reperfusion in groups I, II and IV

Plasmatic creatinine			Creatinine clearance		
I	II	IV	I	II	IV
0,62	1,75	0,46	1,12	0,24	1,16
0,57	0,51	0,27	1,46	1,45	2,79
0,48	1,75	0,39	1,52	0,36	1,71
0,79	0,62	0,39	0,92	2,27	2,28
1,15	0,69	0,38	0,73	1,11	1,29
0,53	0,15	0,41	1,24	4,16	1,36
0,51	0,68	0,55	1,7	1,06	1,09
2,81	0,54	0,46	0,17	1,03	1,61
0,67	0,58	0,5	1,18	0,88	1,55
0,72	0,98	0,4	0,81	0,84	1,41
mean					
0,89	0,83	0,42	1,09	1,34	1,63
Analysis of variance (Kruskal - Wallis test)					
H calc = 13,68 * groups I and II > IV (*)p < 0,05			H calc = 5,65		

Table II - Tubular and interstitial lesional indices in groups I, II, III, IV expressed as mean and median

Index		Group				Analysis variance (Kruskal - Wallis test)	Mann - Whitney test
		I	II	III	IV	(groups I and II x IV)	(groups I x III)
Tubular	Mean Median	2,6 1	2,4 2	4,1 4	0,7 1	H calc = 6,52* I and II > IV p < 0,05	z calc = 1,93 p = 0,280
Interstitial	Mean Median	1,6 1	2,7 3,5	1,1 1	0,1 0	H calc = 13,03* I and II > IV p < 0,01	z calc = 1,06 p < 0,06

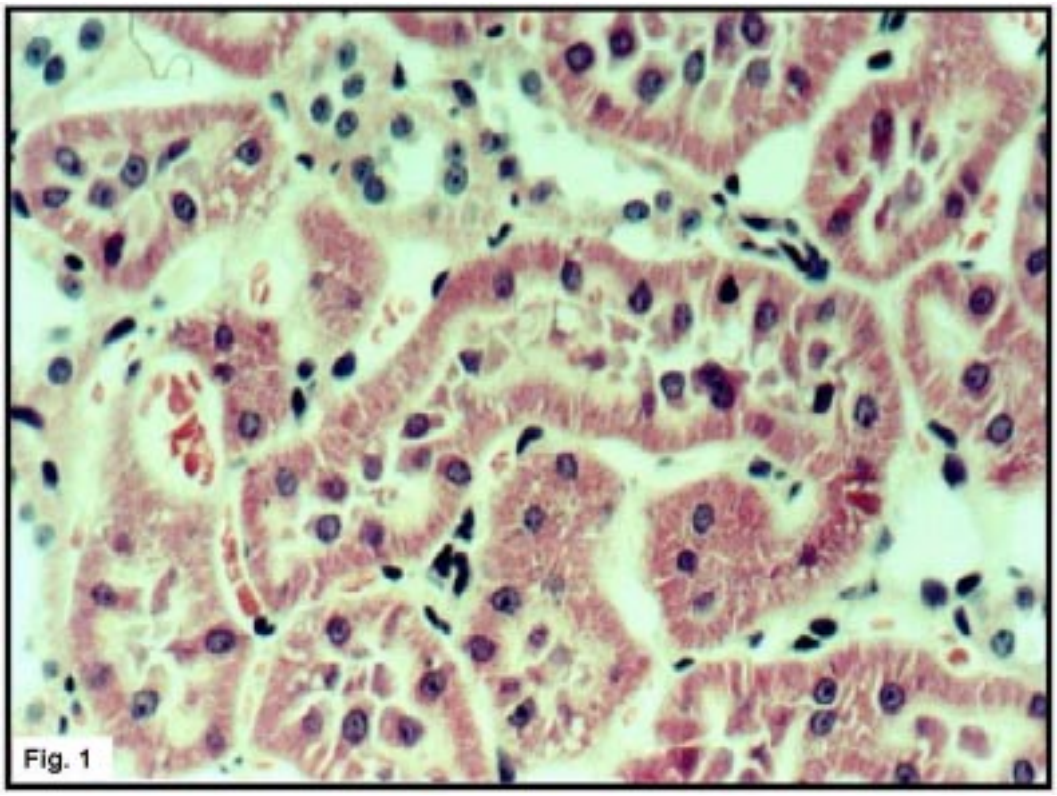

Fig. 1 – Group I: Renal histopathology showing cytoplasmic vacuolization and cell descamation of tubular epithelial cells (HE; 400x).

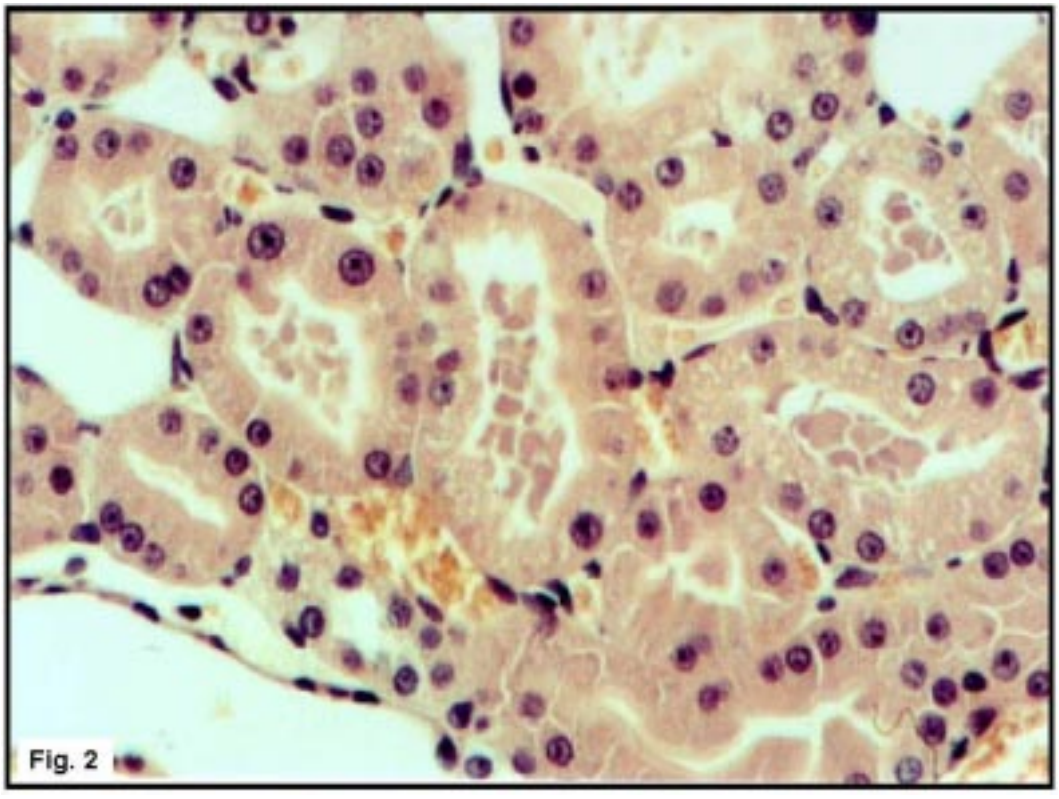

Fig. 2 – Group II: Renal histopathology showing extensive cast formation and interstitial edema (HE; 400x)

Table III - Mean values of groups I, II and III, according to plasmatic creatinine (mg/dl), creatinine clearance (ml/min) and Na urinary/creatinine urinary ratio

Plasmatic creatinine		Creatinine clearance		Na urinary/creatinine urinary ratio	
I	II	I	III	I	III
0,62	0,52	1,12	1,45	0,08	0,14
0,57	0,53	1,16	1,54	0,41	0,18
0,48	0,38	1,52	1,19	0,49	0,3
0,79	0,29	0,92	1,89	0,12	0,2
1,15	0,4	0,73	2,04	0,24	0,18
0,53	0,68	1,24	0,63	0,28	0,17
0,51	0,53	1,7	1,11	0,65	0,12
2,81	0,39	0,17	1,95	0,73	0,12
0,67	0,34	1,18	1,73	0,04	0,11
0,72	0,53	0,81	1,12	0,45	0,11
		mean			
0,89	0,46	1,06	1,47	0,35	0,16
		Mann - Whitney test			
		(groups I x III) U crit = 23			
Plasmatic creatinine U calc.= 15,5 * I > III		Creatinine clearance U calc = 28,5		Na urinary / creat urinary U calc = 16 * I > III	
(*) p < 0,05				(*) p < 0,05	

4- DISCUSSION

In the present model, the abdominal aorta, the bilateral iliac arteries and the branches were dissected and identified. The collateral flow was maintened since the branches of the aorta were not ligated. As rats have a smaller profunda femoral artery, there exist an abundant collateral and reentrant vasculature[4, 20]. These anatomic details were relevant to analyze the severity of hind limb ischemia and renal function in the ischemic-reperfusion injury experimental model. In addition, the model avoids traumatic injury to venous vessels and muscle, and allows the preservation of vascular response during reperfusion, which are factors important to the extension of rhabdomyolysis and renal insufficiency[21,22,12].

We studied the decrease in glomerular filtration resulting from renal hypoperfusion or nephrotoxicity by the evaluation of creatinine concentration, creatinine clearance and the ratio of urine sodium/urine creatinine. There were functional and histological renal alterations during ischemic-reperfusion skeletal muscle injury in 6 and 12 h. The ischemic reperfusion of skeletal muscle injury in all groups was associated with the presence of myoglobin casts in renal tubules. Some experimental and clinical studies have suggested that myoglobin alone does not impair renal function[6]; however, myoglobin nephrotoxicity may occur and may

induce acute renal failure when dehydratation, acidemia or both coexist[6,9,24,25]. Accordingly, it has been generally accepted that myoglobin can exert a nephrotoxic potential when excreted under acid uric conditions. The histologic study showed the presence of tubular obstruction by intraluminal casts, which were composed of swollen blebs of brush border lost from tubular epithelium and by myoglobin[26]. Recent studies using the glycerol model of mioglobinuric acute renal failure suggest that hematin stimulates hydroxyl radical formation producing membrane damage by lipid peroxidation[27,28,29]. In 10 days of reperfusion (group III), the histologic study showed no significant tubular repair. In addition, recent studies suggests that components of the extracelular matrix lead to renal functional and structural alterations[17]. Altogether our data suggest that the ischemic and reperfusion syndrome of hind limbs in rats results in: **i**) Acute renal failure, demonstrated by histological alterations and increase of creatinine plasmatic level; **ii**) No difference in the severity of renal lesions between 6 and 12 h of ischemia, **iii**) Normalization of the creatinine plasmatic levels after 10 days of reperfusion, with persistence of renal histological changes.

TAKITO AM, SILVA JCCB , BUENO V, FRANCO M , BURIHAN E. Síndrome de isquemia e reperfusão dos membros posteriores: estudo funcional e histológico renal em ratos. Medicina (Ribeirão Preto) 2005, 38 (3/4): 294-300.

Resumo: Lesão isquêmica e reperfusão das extremidades pode resultar em síndrome metabólica sistêmica, grave e complexa, caracterizada por insuficiência renal aguda, mioglobinúria, acidose metabólica, produção acentuada de radicais livres e hipercalemia. Neste estudo, investigamos as alterações funcionais e histológicas dos rins após isquemia e reperfusão dos músculos esqueléticos dos membros posteriores. Os ratos foram submetidos à ligadura da aorta infra-renal por 6 e 12 h. Os animais foram randomizados em quatro grupos de 10 ratos: Grupo I, 6 h de isquemia e 24 h de reperfusão; Grupo II, 12 h de isquemia e 24 h de reperfusão; Grupo III, 6 h de isquemia e 10 dias de reperfusão; Grupo IV *sham*, sem isquemia ou reperfusão. Amostras de sangue ao término da experiência e o volume de urina nas primeiras 24 h de reperfusão no grupo I e II; e no último dia no grupo III foram coletadas para análise funcional. Os parâmetros da função renal estudados foram: creatinina plasmática, depuração de creatinina e a relação urinária do sódio urinário/creatinina. Histologicamente, avaliamos semi-quantitativamente, o índice de lesões túbulo-intersticiais. Os resultados evidenciaram aumento dos níveis plasmáticos de creatinina e alterações estruturais nos rins dos animais dos grupos com isquemia e reperfusão. Dez dias após a reperfusão houve recuperação da função renal embora as alterações histológicas ainda persistissem.

Descritores: Isquemia. Reperfusão. Rim.

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Recebido em 31/03/2005

Aprovado em 08/12/2005