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Metabolic acidosis aggravates experimental acute kidney injury

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ABSTRACT

Aims: Ischemia/reperfusion (I/R) injury and metabolic acidosis (MA) are two critical conditions that may simultaneously occur in clinical practice. The result of this combination can be harmful to the kidneys, but this issue has not been thoroughly investigated. The present study evaluated the influence of low systemic pH on various parameters of kidney function in rats that were subjected to an experimental model of renal I/R injury.
Main methods: Metabolic acidosis was induced in male Wistar rats by ingesting ammonium chloride (NH_4Cl) in tap water, beginning 2 days before ischemic insult and maintained during the entire study. Ischemia/reperfusion was induced by clamping both renal arteries for 45 min, followed by 48 h of reperfusion. Four groups were studied: control (subjected to sham surgery, $n = 8$), I/R ($n = 8$), metabolic acidosis (MA; 0.28 M NH_4Cl solution and sham surgery, $n = 6$), and MA + I/R (0.28 M NH_4Cl solution plus I/R, $n = 9$).
Key findings: Compared with I/R rats, MA + I/R rats exhibited higher mortality (50 *vs.* 11%, $p = 0.03$), significant reductions of blood pH, plasma bicarbonate (pBic), and standard base excess (SBE), with a severe decline in the glomerular filtration rate and tubular function. Microscopic tubular injury signals were detected. Immunofluorescence revealed that the combination of MA and I/R markedly increased nuclear factor κB (NF-κB) and heme-oxygenase 1 (HO-1), but it did not interfere with the decrease in endothelial nitric oxide synthase (eNOS) expression that was caused by I/R injury.
Significance: Acute ischemic kidney injury is exacerbated by acidic conditions.

1. Introduction

Acute kidney injury (AKI) remains a tremendous problem in clinical practice, affecting approximately 20% of hospitalized patients and half of critically ill patients who are admitted to intensive care units [1,2,3]. It is also a recurrent condition in allograft survival in patients who undergo kidney transplantation [4]. The pathophysiology of renal injury that is induced by ischemia/reperfusion (I/R) includes myriad events that are not limited to changes in renal hemodynamics and also include tubular damage, the recruitment of inflammatory mediators, and the release of reactive oxygen species [5,6,7]. Because of its multifactorial nature, a better understanding of the underlying mechanisms of AKI and definitive therapeutic approaches to this important clinical situation remain challenging.

Metabolic acidosis (MA) is a frequently encountered acid–base disturbance in critically ill patients [8]. The etiology of MA includes many factors, including lactic acidosis, ketoacidosis, rapid volume expansion with saline, renal failure, and others. Metabolic acidosis can be present in many patients who experience renal injury. Metabolic acidosis increases the presence of inflammatory molecules in experimental sepsis [9]. Moreover, MA can reduce renal blood flow in healthy human volunteers [10] and increase nuclear factor κB (NF-κB) DNA binding and inflammatory mediator release [11]. However, to the best of our knowledge, no study has evaluated the impact of MA on the severity of ischemic AKI. To investigate this issue, the present study examined the impact of MA on renal I/R injury. We hypothesized that MA that is induced by NH_4Cl will increase the severity of I/R injury. In order to elucidate the detrimental effects of MA on rat renal tissues under ischemic AKI, we evaluated the tubular injury and the expression of the endothelial nitric oxide synthase (eNOS), NF-κB and heme-oxygenase 1 (HO-1).

2. Material and methods

2.1. Animals

Male Wistar rats, weighing 270–320 g, were obtained from the central animal facility of the Federal University of Ceará, Fortaleza, Ceará,

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Brazil. Before any procedure, the animals were maintained under standard laboratory conditions (12 h/12 h light/dark cycle) at 22–25 °C with free access to standard chow (Biotec) and tap water. Animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). The study was approved by our Institutional Ethics Committee for Animal Research (CEUA-UFC protocol no. 32/13).

2.2. Metabolic acidosis induction

Metabolic acidosis was induced based on well-established protocols to produce moderate acidosis in response to standard 0.28 M ammonium chloride (NH_4Cl) in drinking water [12]. The animals were individually maintained in metabolic cages for 5 consecutive days. The first 3 days were dedicated to acclimatization. Beginning on the second day, the animals began to drink a solution with 0.28 M NH_4Cl (Vetec, Rio de Janeiro, Brazil). After 48 h of NH_4Cl ingestion, the rats were subjected to surgical procedures to induce renal ischemia (described below). After surgical recovery, the animals were placed again in individual metabolic cages, and NH_4Cl ingestion was maintained for an additional 2 days. Control animals ingested only tap water for 5 days and underwent the same surgical procedures, with the exception of renal ischemia induction (see below). All of the animals were provided standard chow *ad libitum* during the first 4 days of experimentation, with the exception of the 12 h period before the renal I/R surgical procedures. On the last day, the animals were fasted. Fluid intake and urine output were measured every 24 h.

2.3. Surgical procedures for induction of renal ischemia/reperfusion

Forty-eight hours after the induction of MA, each rat was weighed and anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A midline laparotomy incision was made, and ischemic renal failure was induced by clamping both renal arteries with a non-traumatic clamp for 45 min, followed by reperfusion. The procedure was performed on a heated pad to maintain body temperature at 37 °C. After 48 h, mean arterial pressure was measured through a catheter that was inserted in the carotid artery, and the animals were sacrificed to obtain blood samples for the biochemical tests. Heart rate was estimated based on pulse-pressure traces. The left kidneys were collected for histological and immunofluorescent evaluation.

2.4. Experimental groups

The rats were randomly divided into the following groups: control (CTL; $n = 8$; free access to tap water and subjected to sham surgical procedures without bilateral clamping of the renal arteries), MA ($n = 6$: free access to NH_4Cl solution instead of tap water for 48 h before and after the surgical procedures and subjected to sham surgical procedures without bilateral clamping of the renal arteries), I/R ($n = 8$; free access to tap water and subjected to bilateral clamping of the renal arteries for 45 min, followed by reperfusion), MA + I/R ($n = 9$; free access to NH_4Cl solution instead of tap water for 48 h before and after the surgical procedures and subjected to bilateral clamping of the renal arteries for 45 min, followed by reperfusion).

2.5. Measurement of biochemical parameters

Immediately after collection, blood samples were analyzed using a gasometer device (COBAS B 121, Roche, Mannheim, Germany) to determine arterial blood gas, hematocrit, and osmolality. The blood samples were centrifuged at 3500 rotations per minute (rpm) for 10 min at 4 °C to obtain plasma, which was maintained at −80 °C for subsequent creatinine (Cr), urea (BUN), and electrolyte (Na^+, K^+, and Cl) determination. Twenty-four-hour urine samples were collected in light-protected bottles under mineral oil at room temperature (22–25 °C). After removing the oil phase, the urine samples were centrifuged at 3000 rpm for 5 min at room temperature and pH was measured immediately using pHmeter (Marte, MD-10, São Paulo, Brazil). Subsequently, the resulting supernatant was maintained at −80 °C until further analysis (creatinine, urea and electrolytes). Plasma and urine Cr and BUN levels were determined by colorimetry using a spectrophotometer (Bio Plus, Bio 200, São Paulo, Brazil) and commercial kits (Labtest Diagnostics, Minas Gerais, Brazil). Plasma and urine Na^+, K^+, and Cl^- levels were determined using ion-selective electrodes (Model 9180, Roche, Mannheim, Germany). These values allowed us to estimate creatinine clearance (CrCl), which is used as an indicator of the glomerular filtration rate (GFR) and fractional excretion of sodium (FE_{Na+}), potassium (FE_{K+}), and chloride (FE_{Cl^-}). All assessments were blinded.

2.6. Histological analysis

Renal tissue was fixed in 10% buffered formalin for 24 h and then maintained in 70% alcohol for subsequent processing in paraffin for the histological and immunofluorescence studies. Slices (4 μm thick) were obtained and stained with hematoxylin and eosin. The sections were observed by light microscopy (400× magnification) to assess the presence of tubular cell necrosis, tubular dilation, inflammatory cell infiltration, and cellular edema in the tubular interstitium from 10 non-overlapping fields that were randomly selected in the renal cortex and outer medulla for each animal in a blinded manner. Tissue damage is expressed as a percentage of affected kidney samples using a semiquantitative scale: 1 (mild lesions that affected <10% of kidney samples), 2 (lesions that affected 10–25% of kidney samples), 3 (lesions that affected 26–50% of kidney samples), 4 (lesions that affected 51–75% of kidney samples), and 5 (lesions that affected more than 75% of kidney samples).

2.7. Expression of eNOS, NF-κB, and HO-1

Deparaffinized kidney sections (4 μm thick) were incubated with primary antibodies (endothelial nitric oxide synthase [eNOS; rabbit polyclonal; sc-654, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:200], NF-κB [rabbit polyclonal; sc-114, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:200], and HO-1 [goat polyclonal; sc-1796, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:200]) overnight at 4 °C. The sections were then incubated with secondary antibodies (Alexa Fluor 568 conjugated to anti-rabbit IgG antibody [1:400, Invitrogen, A10042] or Alexa Fluor 594 conjugated to anti-goat IgG antibody [1:400, Invitrogen, A11058]) for 90 min at room temperature in a darkened humidified chamber. Finally, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted on slides, and observed in a confocal laser scanning microscope (LSM 710 Zeiss, Munich, Germany). Negative controls were processed as described above but incubated with 5% bovine serum albumin in phosphate-buffered saline instead of the primary antibody, and no specific staining was performed. Ten non-overlapping fields were randomly selected for each animal in the corticomedullary region (400× magnification). Fluorescence intensity was analyzed using FIJI-Image J software, and the number of pixels in the selected area was estimated from the total number of pixels in the entire image.

2.8. Force measurement in isolated renal artery

In a set of animals, the left renal artery was removed to record endothelium-dependent vasorelaxant effects that were induced by acetylcholine (ACh, Sigma-Aldrich, St Louis, MO, USA). Ring-like segments (~1.5 mm length) were mounted on a myograph system (610 M-DMT, Danish Myo Technology A/S, Aarhus, Denmark) under 5 mN resting tension. The nutrient medium was Krebs–Henseleit solution (37 °C, 95% O_2/5% CO_2, pH 7.4). Concentration–response curves were

Table 1
Acid–base and hemodynamic variables according to experimental group.

	CTL	I/R	MA	MA + I/R
Number of animals	8	8	6	9
Body weight, g				
Day 0	287 ± 4	282 ± 3	288 ± 9	294 ± 6
Day 5	257 ± 5[d]	256 ± 6[d]	251 ± 7[d]	258 ± 4[d]
Blood				
pH	7.39 ± 0.01	7.35 ± 0.03	7.27 ± 0.04[a]	7.00 ± 0.04[a,b,c]
pBic, mmol/l	23.4 ± 0.4	21.4 ± 0.9	19.0 ± 1.7[a]	9.0 ± 1.4[a,b,c]
SBE, mmol/l	−0.8 ± 0.5	−2.7 ± 0.9	−6.8 ± 2.4[a]	−23.8 ± 1.5[a,b,c]
Hematocrit, %	43.0 ± 0.8	40.1 ± 0.8	45.0 ± 0.5[b]	42.3 ± 1.7
Osmolality, mosm/kg H_2O	281.5 ± 0.7	277.7 ± 1.8	282.3 ± 0.9	290.6 ± 5.1[b]
Hemodynamic indices				
MAP, mm Hg	109 ± 6	100 ± 5	91 ± 4	95 ± 7
HR, beats/min	400 ± 9	363 ± 11	400 ± 9	364 ± 13

CTL, control (subjected to sham surgery); I/R, ischemia/reperfusion injury (tap water and subjected to bilateral renal ischemia for 45 min); MA, metabolic acidosis (0.28 M NH_4Cl solution and sham surgery), and MA + I/R, metabolic acidosis plus ischemia/reperfusion (0.28 M NH_4Cl solution and subjected to bilateral renal ischemia for 45 min). The data are expressed as the mean ± SEM on the last day of the experiment. pBic, plasma bicarbonate; SBE, standard base excess; MAP, mean arterial pressure; HR, heart rate.
[a] $p < 0.05$, *vs.* CTL.
[b] $p < 0.05$, *vs.* I/R.
[c] $p < 0.05$, *vs.* MA.
[d] $p < 0.05$, *vs.* Day 0 (Student's paired *t*-test).

constructed for 0.01–30 μM ACh using steady-state contractions that were elicited by 1 μM phenylephrine (PE, Sigma-Aldrich, St Louis, MO, USA).

2.9. Statistical analysis

All of the data are expressed as mean ± SEM. Comparisons among groups were performed using Student's paired *t*-test and one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls or Holm–Sidak *post hoc* test as appropriate. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mortality

During experimental procedures, a total number of 10 rats died (1 from the I/R group [11% of mortality] and 9 from the MA + I/R group [50% of mortality], $p = 0.03$) during the experimental period (between 12 and 24 h after the surgical procedures). Data collected from these animals were not included in the analyzed data. CTL and MA groups showed no mortality under our experimental condition.

3.2. Metabolic acidosis and hemodynamic parameters

Table 1 summarizes the functional parameters of rats that were subjected to MA induction with or without I/R. Although all of the animals presented a significant reduction of body weight during the experimental period, no significant differences in weight loss were observed between groups. As expected, the MA group had lower blood pH, plasma bicarbonate (pBic), and standard base excess (SBE) compared with the CTL group, and the degree of MA was more severe in the MA + I/R group. No significant differences in pH, pBic, or SBE were found between the CTL and I/R groups. No differences in mean arterial pressure or heart rate were found between groups.

3.3. Metabolic acidosis worsened I/R-induced functional impairment

Animals that were subjected to I/R injury exhibited increases in the plasma levels of Cr (2.9 ± 0.7 *vs.* 0.6 ± 0.03 mg/dl, $p < 0.05$) and urea (176.4 ± 22.9 *vs.* 36.5 ± 1.6 mg/dl, $p < 0.05$), reflecting a marked reduction of CrCl (0.14 ± 0.03 *vs.* 0.35 ± 0.02 ml/min/100 g, $p < 0.05$) compared with the CTL group. This reduction of CrCl was associated with high Na^+ excretion (2.36 ± 0.72% *vs.* 0.26 ± 0.04%, $p < 0.05$). The animals that received NH_4Cl solution without I/R (MA group) exhibited

Table 2
Parameters of renal function in rats subjected to NH_4Cl-induced metabolic acidosis and renal ischemia/reperfusion.

	CTL	I/R	MA	MA + I/R
Plasma				
Creatinine, mg/dl	0.6 ± 0.03	2.9 ± 0.70[a]	0.7 ± 0.02[b]	5.8 ± 0.9[a,b,c]
Urea, mg/dl	36.5 ± 1.6	176.4 ± 22.9[a]	50.5 ± 1.7[b]	355.2 ± 61.4[a,b,c]
$[Na^+]$, mmol/l	136.9 ± 1.0	136.6 ± 1.5	137.5 ± 0.7	146.0 ± 2.4[a,b,c]
$[K^+]$, mmol/l	3.9 ± 0.1	4.1 ± 0.3	4.1 ± 0.2	7.0 ± 0.5[a,b,c]
$[Cl^-]$, mmol/l	105.8 ± 0.5	103.3 ± 1.1	111.3 ± 2.3[b]	120.8 ± 2.6[a,b,c]
CrCl, ml/min/100 g	0.35 ± 0.02	0.14 ± 0.03[a]	0.33 ± 0.03[b]	0.05 ± 0.02[a,b,c]
Urine				
pH	6.83 ± 0.08	6.26 ± 0.35	5.69 ± 0.11	6.65 ± 0.31
FE_{Na+}, %	0.26 ± 0.04	2.36 ± 0.72[a]	0.25 ± 0.05[b]	7.17 ± 1.82[a,b,c]
FE_{K+}, %	22.67 ± 2.17	101.19 ± 31.28[a]	15.81 ± 0.68[b]	99.41 ± 13.08[a,c]
FE_{Cl-}, %	0.59 ± 0.08	4.52 ± 1.60[a]	0.80 ± 0.14[b]	16.77 ± 5.11[a,b,c]

CTL, control (subjected to sham surgery); I/R, ischemia/reperfusion injury (tap water and subjected to bilateral renal ischemia for 45 min); MA, metabolic acidosis (0.28 M NH_4Cl solution and sham surgery), and MA + I/R, metabolic acidosis plus ischemia/reperfusion (0.28 M NH_4Cl solution and subjected to bilateral renal ischemia for 45 min). The data are expressed as the mean ± SEM on the last day of the experiment. CrCl, creatinine clearance; FE_{Na+}, FE_{K+}, and FE_{Cl-}, fractional excretion of Na^+, K^+, and Cl^-, respectively.
[a] $p < 0.05$, *vs.* CTL.
[b] $p < 0.05$, *vs.* I/R.
[c] $p < 0.05$, *vs.* MA.

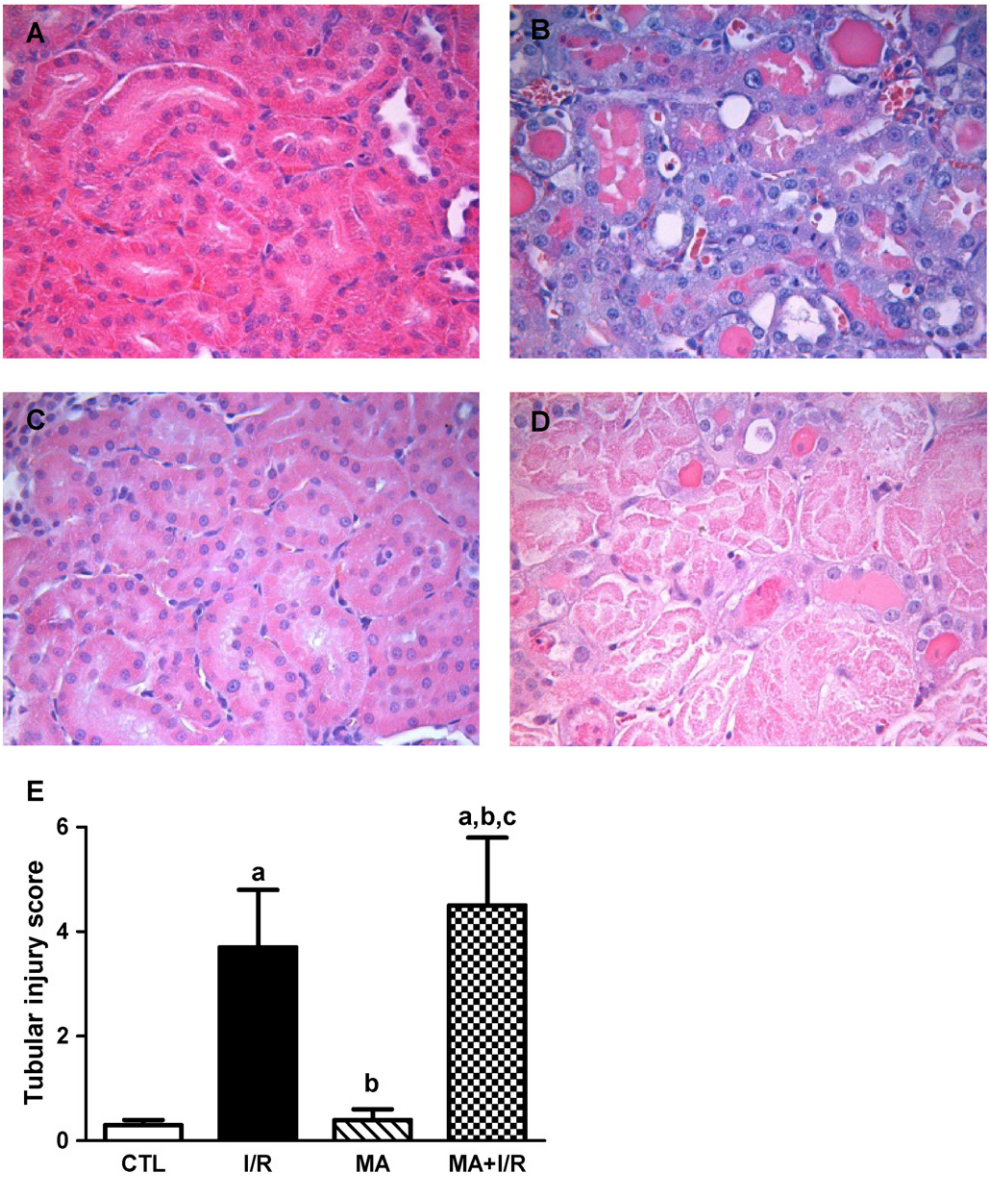

Fig. 1. Morphological changes in rat kidneys subjected to MA and I/R injury. Photomicrographs of kidney tissues obtained from rats in the (A) control group, (B) ischemia/reperfusion (I/R) injury group, (C) NH_4Cl-induced metabolic acidosis (MA) group, and (D) NH_4Cl-induced metabolic acidosis plus I/R injury (MA + I/R) group. In A, the tissue presented a preserved renal structure, whereas the kidney in C presented mild tubular dilatation and vacuolation in the renal epithelia. In B, necrotic renal tubules with a hyaline cast inside the lumen of the renal tubule can be seen. In D, one can see intense tubular necrosis (hematoxylin and eosin staining, 400× magnification). (E) Tubular injury score. The data are expressed as mean ± SEM. $^{a}p < 0.05$, *vs.* CTL; $^{b}p < 0.05$, *vs.* I/R; $^{c}p < 0.05$, *vs.* MA.

no alterations in these parameters compared with the control group, and MA induction worsened the functional alterations that were induced by I/R injury. The MA + I/R group had higher Cr and urea compared with the I/R group. A significant reduction of CrCl was observed in the MA + I/R group compared with the I/R group (0.05 ± 0.02 *vs.* 0.14 ± 0.03 ml/min/100 g, $p < 0.05$). This severe functional impairment was reflected by a substantial increment of the fractional excretion of Na^+ (Table 2).

3.4. Metabolic acidosis aggravated histological injury

The histological analysis of the kidney samples in the CTL and MA groups showed only mild alterations in tubules, whereas rats that were subjected to I/R injury exhibited tubular necrosis, tubular dilation, inflammatory cell infiltration, and cellular edema in the tubular interstitium of the renal cortex and outer medulla. These conditions were worsened in the MA + I/R group. Representative photographs and the semiquantitative analysis of renal damage in each experimental group are shown in Fig. 1.

3.5. Metabolic acidosis did not affect the ischemia/reperfusion-induced downregulation of eNOS or responsiveness of renal artery to acetylcholine

Forty-eight hours after the surgical procedures, the I/R group exhibited markedly lower eNOS protein expression compared with the CTL group ($p < 0.05$). Animals that were subjected only to MA had similar eNOS expression as animals in the I/R group. In animals that were subjected to both MA and I/R, the reduction of eNOS expression was not significantly different from the I/R group ($p > 0.05$; Fig. 2, accompanied by a representative image of confocal fluorescence microscopy).

Functional studies in isolated rings of the renal artery revealed that MA did not influence the relaxant action of ACh (0.01–30 μM) against PE-induced contractions (Fig. 3). In contrast, cholinergic relaxation significantly decreased in the renal arteries in rats that were subjected to I/

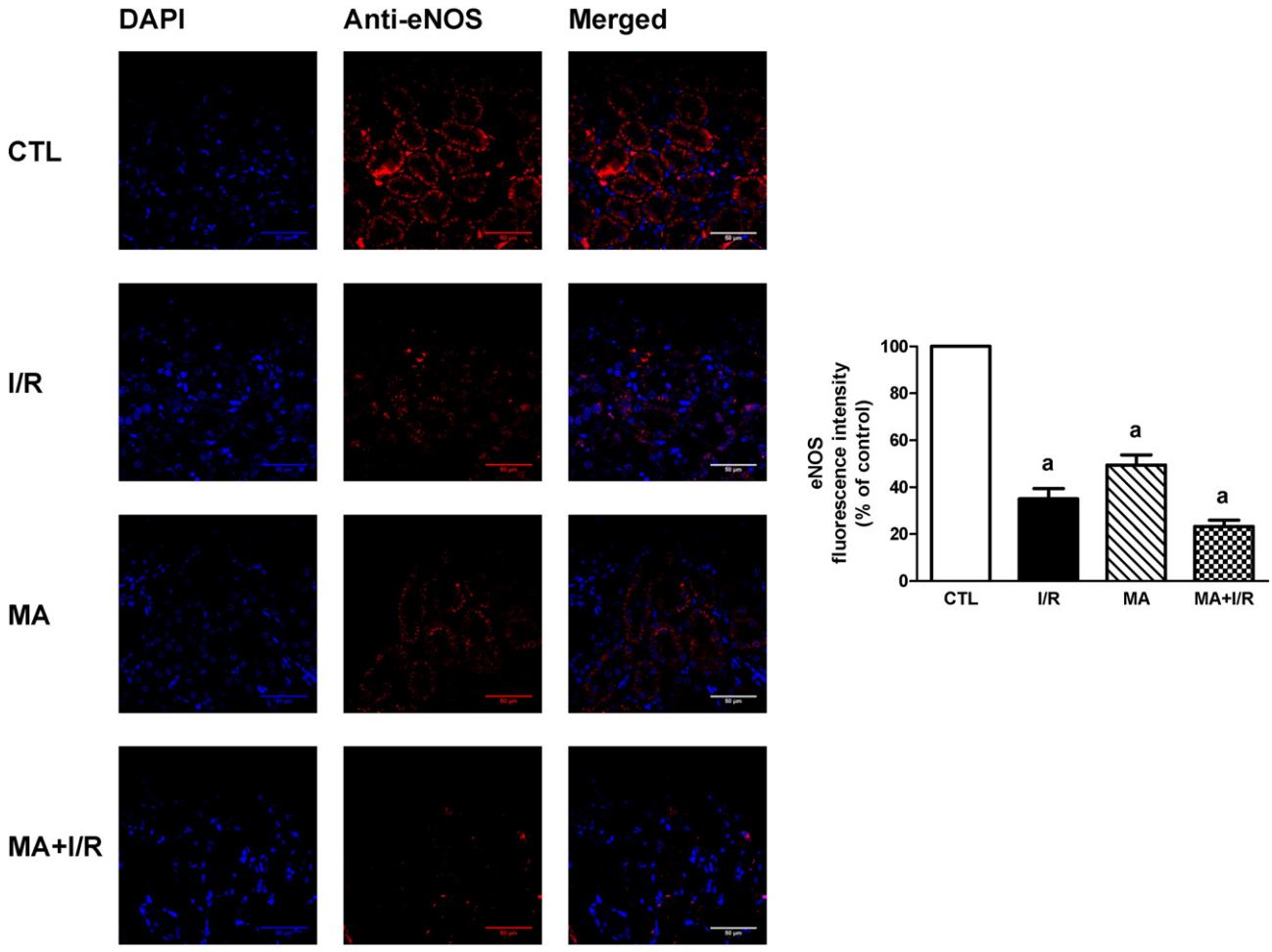

Fig. 2. Effect of MA and I/R on eNOS expression. The images reveal significantly less immunofluorescence related to eNOS in tubular cells in response to acid load or I/R, a phenomenon that did not increase when both insults (MA and I/R) were present together. Scale bar = 50 μm. The data are mean ± SEM ($n = 6$) expressed as percentage of the values obtained to CTL group. [a]$p < 0.05$, *vs.* CTL.

R. Acetylcholine reduced PE-induced contractions to only 68.8 ± 3.4% and 67.5 ± 9.1% of control values in the I/R and MA + I/R groups, respectively. These values were significantly different from the CTL group (28.8 ± 4.3%, $p < 0.05$).

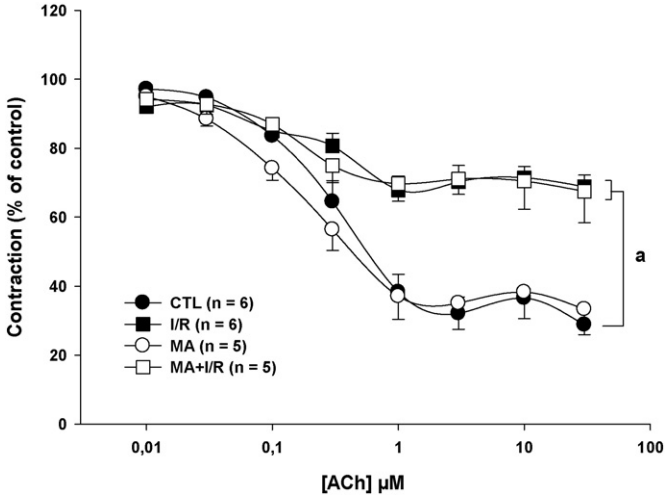

Fig. 3. Vasorelaxation induced by ACh in the renal artery in rats subjected to MA and I/R. Isolated vessels were precontracted with 1 μM phenylephrine (PE). Acetylcholine-induced relaxation was significantly attenuated in vessels from I/R (□) and MA + I/R rats (□) but not in MA rats (○) compared with control (CTL, ●) tissues. The data are expressed as mean ± SEM. The number of rats is shown in parentheses. [a]$p < 0.05$, *vs.* CTL.

3.6. Metabolic acidosis augmented the ischemia/reperfusion-associated up-regulation of NF-κB

Animals that were subjected only to MA or I/R had similar NF-κB expression, although NF-κB expression increased in both groups compared with the CTL group ($p < 0.05$). When combined with I/R, MA further increased NF-κB expression compared with the I/R group ($p < 0.05$; Fig. 4).

3.7. Metabolic acidosis augmented the ischemia/reperfusion-induced up-regulation of HO-1

As shown in Fig. 5, a significant increase in HO-1 expression was observed in the MA group compared with the CTL group ($p < 0.05$). This increase was similar to the I/R group ($p < 0.05$). Animals in the MA + I/R group had even higher levels of HO-1 expression than the I/R group ($p < 0.05$).

4. Discussion

In the present study, we found that prior MA worsened renal I/R injury, reflected by a greater increase in NF-κB expression but no additive effect on eNOS protein expression, which was decreased by I/R and occurred even under greater expression of HO-1, a known mechanism of protection against I/R injury. Also, MA increased the mortality early (12-24 h) after I/R procedure in comparison with animals submitted only to I/R.

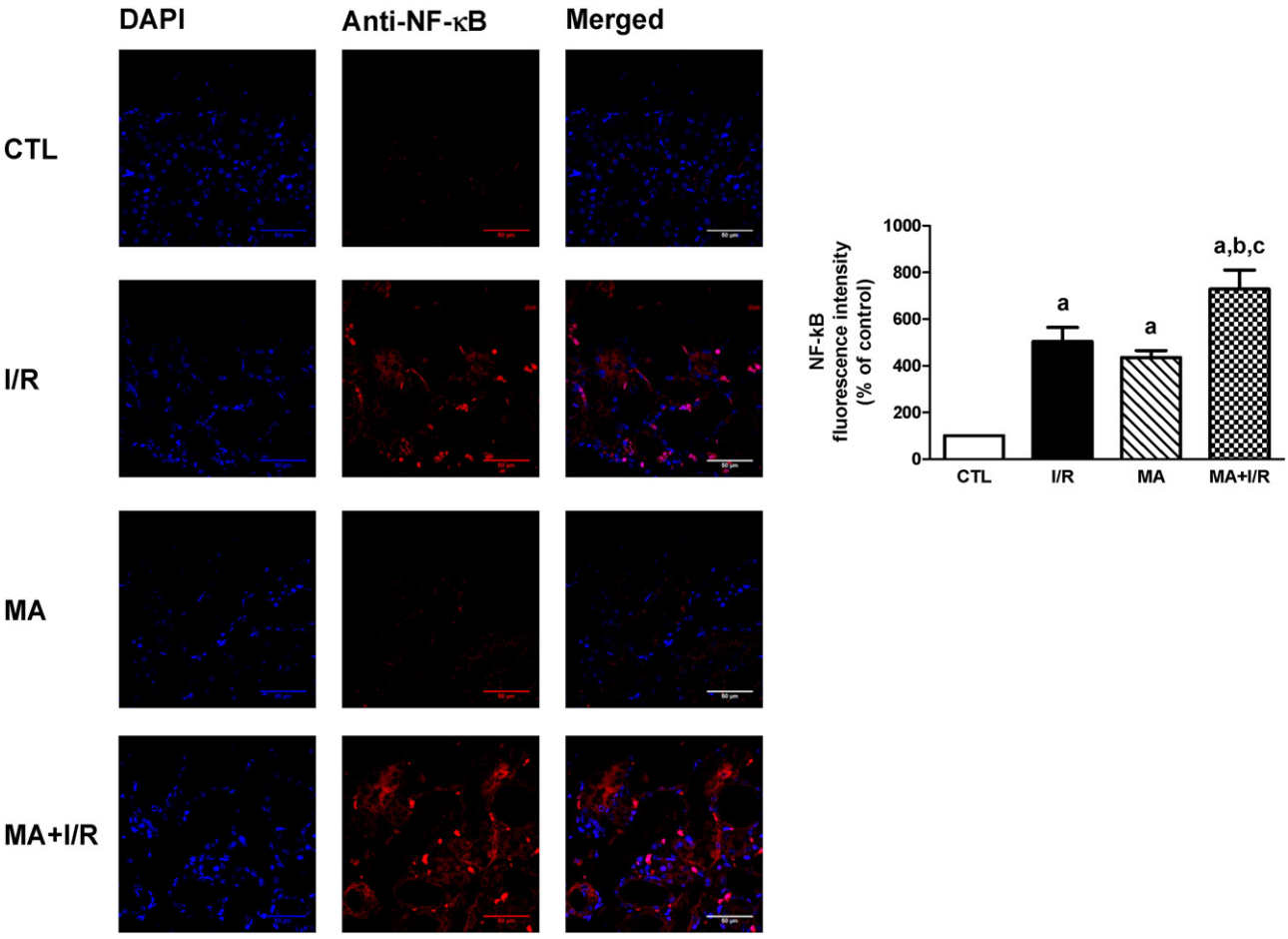

Fig. 4. Effect of MA and I/R on NF-κB expression. Compared with CTL rats, the intensity of NF-κB fluorescence significantly increased in the I/R and MA groups. This increase was intensified when MA occurred simultaneously with I/R-induced renal injury. Scale bar = 50 μm. The data are mean ± SEM ($n = 6$) expressed as percentage of the values obtained to CTL group. $^{a}p < 0.05$, *vs.* CTL; $^{b}p < 0.05$, *vs.* I/R; $^{c}p < 0.05$, *vs.* MA.

Acute kidney injury has a complex pathogenesis and different stages that can include endothelial damage and inflammation, which can culminate in organ failure [13]. Metabolic acidosis can have deleterious effects on the kidneys and involves similar processes, including inflammation and changes in renal blood flow. Although MA is a known consequence of severe kidney impairment, many critically ill patients have MA from other causes (*e.g.*, lactic acidosis and hyperchloremic acidosis due to volume expansion), even before AKI is initiated. Although our data cannot be extrapolated to lactic acidosis, we can suggest that hyperchloremic acidosis developing in critically ill patients can be harmful, favoring the use of balanced solutions in volume expansion [14]. It is difficult to ascertain that acidosis treatment with alkali therapy can arrest the detrimental effect of metabolic acidosis but this must be considered.

In the present study, we subjected a group of animals to MA 48 h before I/R injury. Using this approach, the animals in the MA group developed a moderate degree of MA. The induction of MA with NH_4Cl can slightly induce dehydration in experimental rats [15], and this was one major caveat that we considered in our study. However, dehydration was unlikely because both the CTL and experimental animals presented similar decreases in body weight at the end of the experimental procedures. The observed decrease in body weight of control and experimental animals can be attributed to acclimatization period in metabolic cage. The reduction in animal body weight in single metabolic cage may be due to increased levels of circulating corticosterone [16].

Additionally, mean arterial pressure and heart rate did not differ from CTL rats. Thus, damage to renal structures appeared to be attributable to the systemic influence of acid–base status and not macrohemodynamic parameters.

Although MA-induced exacerbation of the inflammatory response is already known, no consensus has been reached regarding the effects of such acid–base imbalance in I/R organ injury. Some studies have even suggested a protective effect of milder forms of acidosis (metabolic or hypercapnic) in other organs, such as the lungs, heart, and brain [17,18,19]. In the present study, the animals developed moderate MA before applying the I/R procedure, and MA became more severe 48 h after I/R injury. Nevertheless, the possibility that less severe acidosis can produce an opposite effect under other conditions cannot be discarded.

Inducible NOS (iNOS) is fundamentally involved in the process of renal damage and induces inflammation and apoptosis. The inhibition of iNOS activity (or the absence of iNOS itself in knockout mice) improves renal I/R damage *in vivo*. Another isoform of NOS, eNOS, has protective effects on I/R injury [20]. The present study found significantly lower eNOS expression in response to renal I/R, which is supported by previous studies [21,22]. Although studies on the effects of acidic pH on tubular eNOS expression are still scarce, Giraldez et al. [23] reported that I/R-induced acidosis can reduce eNOS expression in heart tissue. In the present study, systemic acidosis alone significantly reduced tubular eNOS expression. With MA and I/R combined, eNOS expression remained comparable to the reduced levels that were induced solely by acid-load.

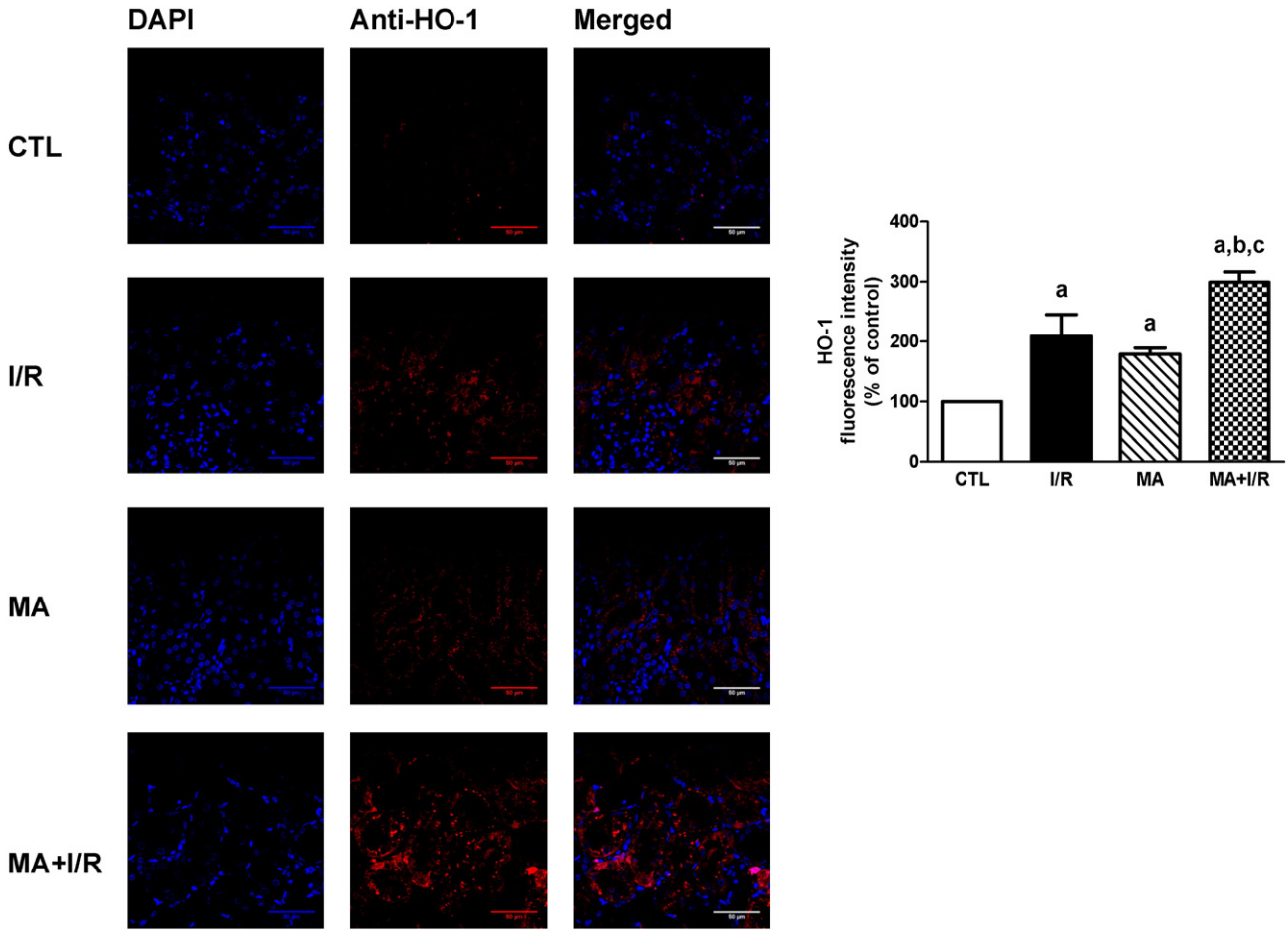

Fig. 5. Effect of MA and I/R on HO-1 expression. Microphotographs revealed that immunofluorescence related to HO-1 increased in tubular cells from I/R and MA rats compared with controls, with a further significant increase in fluorescence in the MA + I/R group. Scale bar = 50 μm. The data are mean ± SEM ($n = 6$) expressed as percentage of the values obtained to CTL group. $^{a}p < 0.05$, *vs.* CTL; $^{b}p < 0.05$, *vs.* I/R; $^{c}p < 0.05$, *vs.* MA.

Additional support for these data may be derived from the vasorelaxant effects of ACh in renal artery rings. The endothelium-dependent relaxant response to ACh is known to be mediated by NO release through an excitatory action on eNOS [24]. Thus, in the present study, we observed a reduction of the ability of ACh to induce endothelium-dependent vasorelaxation, which was significantly more evident in isolated vessels that were obtained from I/R and MA + I/R rats. In renal vessels that were isolated from I/R rats, eNOS underwent a functional loss in endothelial cells, although acidosis did not interfere *per se* with the responsiveness of the renal arteries to ACh.

NF-κB is a key mediator of acute and chronic inflammation. It is considered essential in the induction of I/R injury because it can upregulate proinflammatory gene expression [25]. In the present study, the magnitude of NF-κB upregulation in acidotic rats was comparable to animals that were subjected solely to I/R injury. In contrast, acidotic rats that presented I/R injury exhibited greater NF-κB expression than animals that were subjected only to isolated renal insult (I/R or MA). Hypercapnic acidosis appears to inhibit NF-κB expression in the lungs under conditions of I/R injury [26]. Our data indicate that severe I/R injury combined with MA is associated with high NF-κB expression, suggesting that other factors beyond solely pH can influence NF-κB activity.

Investigators have recently reported enhanced HO-1 expression under conditions of oxidative stress, providing evidence that the end products of heme degradation, including biliverdin, bilirubin, and carbon monoxide, can exert protective actions against renal I/R injury through antioxidant, antiinflammatory, and cytoprotective effects [27]. Rats in the MA group exhibited an increase in HO-1 expression. Interestingly, the MA + I/R group exhibited an increase in HO-1 expression compared with I/R animals. A plausible explanation for this augmented expression may involve the putative release of free heme from destabilized heme proteins in injured cells [28,29]. The increase in HO-1 expression appears to be an attempt to alleviate renal injury that is exacerbated by inflammatory and vascular alterations. Additionally, hypercapnic acidosis upregulated HO-1 expression in lung I/R injury [30].

The present study has several limitations. First, we did not evaluate the effects of different intensities of MA, which could have revealed whether milder acidosis produces different effects. Second, we cannot extrapolate our findings to lactic acidosis, a common etiology of metabolic acidosis in clinical setting. Also, it is possible the MA can also affect the cardiovascular function, which may exacerbate the renal injury, in addition to its direct effect. Finally, the present experimental approach cannot elucidate whether other pathways (*e.g.*, oxidative stress) that are involved in establishing MA can worsen renal I/R injury, and this issue deserves further investigation.

5. Conclusion

The present results showed that MA exacerbates renal injury that is induced by I/R, a phenomenon that was associated with high NK-κB expression. This detrimental event occurred even when protection afforded by HO-1 was maintained.

Conflict of interest

The authors declare that there are no conflicts of interest.

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