Prevention of renal ischemia/reperfusion injury in rats using acetylcysteine after anesthesia with isoflurane[1]

Prevenção de lesão de isquemia/reperfusão em ratos com acetilcisteína após anestesia com isoflurano

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ABSTRACT

PURPOSE: To evaluate the effect of N-acetylcysteine, as a renoprotective agent, when administered early after anesthesia induction, against ischemia/reperfusion injury in rats anesthetized with isoflurane.

METHODS: Eighteen male Wistar rats weighing > 300g were anesthetized with isoflurane. The internal jugular vein and the left carotid artery were dissected and cannulated. The animals were randomly divided into GAcetyl, receiving intravenous N-acetylcysteine, 300mg/kg, and GIsot, isotonic saline. After 30 minutes, right nephrectomy was performed and the left renal artery was clamped during 45 minutes. The animals were sacrificed after 48 hours and blood samples were taken after anesthetic induction and upon sacrificing of the animals to evaluate blood creatinine. The kidneys were sent for histological analysis.

RESULTS: The variation in serum creatinine was 2.33mg/dL ± 2.21 in GAcetyl and 4.38mg/dL ± 2.13 in GIsot (p=0.074). Two animals presented intense tubular necrosis in GAcetyl, compared to 5 in GIsot. Only GAcetyl presented animals free of tubular necrosis (two) and tubular degeneration (one). **CONCLUSION**: After renal ischemia/reperfusion, the rats which were given N-acetylcysteine presented less variation in serum creatinine and milder kidney injuries than the control group.

Key words: Isoflurane. Acetylcysteine. Acute Kidney Injury. Rats, Wistar.

RESUMO

OBJETIVO: Avaliar o efeito da N-acetilcisteína na proteção renal contra lesão de isquemia/reperfusão, quando administrada logo após a indução anestésica, em ratos anestesiados com isoflurano.

MÉTODOS: Dezoito ratos Wistar machos pesando mais que 300g foram anestesiados com isoflurano. A jugular interna direita e a carótida esquerda foram dissecadas e canuladas. Os animais foram distribuídos aleatoriamente em GAcetil, recebendo N-acetilcisteína por via intravenosa, 300mg/kg, e GIsot, solução salina. Foi realizada nefrectomia direita e clampeamento da artéria renal esquerda por 45 min. Os animais foram sacrificados após 48h, sendo colhidas amostras sanguíneas após a indução anestésica e ao sacrifício dos mesmos para avaliar a creatinina sérica. Realizou-se histologia renal.

RESULTADOS: A variação da creatinina foi 2,33mg/dL ± 2,21 no GAcetil e 4,38mg/dL ± 2,13 no GIsot (p=0,074). Dois animais apresentaram necrose tubular intensa no GAcetil, comparados a cinco no GIsot. Apenas GAcetil apresentou animais livres de necrose tubular (dois) e degeneração tubular (um).

CONCLUSÃO: Após isquemia/reperfusão renais, os ratos aos quais se administrou N-acetilcisteína apresentaram menor variação na creatinina sérica e lesões renais mais leves que o grupo controle.

Descritores: Isoflurano. Acetilcisteína. Lesão Renal Aguda. Ratos Wistar.

Introduction

Acute renal failure (ARF) has high incidence in intensive care units, representing an isolated prognostic factor in patients with multiple organ and system disorder[1].

Reactive oxygen species are normally produced during cell metabolism. Free radicals may affect cell vitality by several mechanisms, interfering in the cell growth and proliferation, regeneration and tissue repair, inflammatory response and immune process. The main compounds are the superoxide anion, hydrogen peroxide and the hydroxyl radical. Nitric oxide and the peroxynitrite radical also present the capacity to induce oxidation damage[2]. Kidneys are responsible for 10% of the body oxygen consumption. They are physiological sources of free radicals, especially stemming from the tubular metabolism[3]. This production increases during the tissue insult process and may contribute towards the installation and progression of the kidney injury[4].

ARF incidence during the postoperative period varies in accordance with the series studied and the established definition. Kidney dysfunction is associated with worst case prognoses, especially when dialysis is need. A review conducted on patients who underwent vascular surgery revealed an incidence ranging between 1.7% and 25%[5]. The risk factors can be related to the patient, such as pre-existing kidney or heart dysfunction, diabetes, sepsis, liver failure and exposure to nephrotoxins, or to the surgical procedure, such as procedures involving extracorporeal circulation or aortic clamping, surgeries that result in an increase in intra-abdominal pressure, kidney or liver transplants and use of intravenous contrasts[6].

Several drugs have been used, without success, in the attempt to prevent ARF in risk situations, such as atrial natriuretic peptide[7], calcium channel blockers[8], dopamine[9], loop diuretics[10], mannitol[11], among others. The studies will therefore continue in order to find agents that benefit more serious situations. N-acetylcysteine is a thiol with mucolytic properties that is broadly used in clinical practice with few side effects. It has direct and indirect antioxidant properties, a precursor for L-cysteine and glutathione[12], a source of sulfhydryl groups and free radical scanner[13]. It has been studied in prophylaxis of nephropathy induced by contrast[14] and in kidney[15,16], lung[17] and intestinal reperfusion injury[18], still with conflicting results. It has been administered to animals by intra-peritoneal injection, before ischemia and in different doses, as 200mg/kg, 24 and 12 hours before[15], 200mg/kg, 24, 12 and two hours before[16], and 40 or 300mg/kg, 24 hours and 15 minutes before[19], usually with 45 minutes of arterial clamping. Recent studies revealed kidney reperfusion injuries with only 25 minutes of clamping[20]. It has yet to be determined whether it will have beneficial effect if administered at the beginning of the anesthetic-surgical procedure.

The aim of this study was to evaluate the effect of N-acetylcysteine as a renoprotective agent, when administered early after induction, against ischemia/reperfusion injury in rats anesthetized with isoflurane.

Methods

This study was approved by the Committee for Ethics in Animal Experimentation at the Botucatu School of Medicine, UNESP. Eighteen male Wistar rats weighing more than 300g were anesthetized with isoflurane in an appropriate campanula. After anesthesia induction, they were intubated with an orotracheal cannula and maintained in controlled mechanical ventilation (Harvard Rodent Ventilator, USA). Anesthesia was maintained with isoflurane in 0.3 FIO_2 (Ohmeda vaporizer, USA). The right internal jugular and the left carotid artery were dissected and cannulated with 24G venocath. At that moment, 1mL of blood was collected for the serum creatinine determination, which was replaced with 2mL of isotonic saline solution. An isotonic saline solution infusion was maintained at 5mL/kg/h with an infusion pump (Anne Pump, Abbott, USA). Rectal temperature was maintained between 36°C and 38°C using a thermal bag under and on the animal. Mean arterial pressure (MAP1, before clamping, and MAP2, immediately after clamping the left renal artery), O_2 saturation ($SatO_2$) (sensor on the animal's tongue), end expired CO_2 (ET_{CO2}) and the expired isoflurane fraction (ET_{iso}) (recorded every 15 minutes until the end of the experiment) were monitored by the Datex Engstron, Finland. The animals were divided into two groups using randomizing computer software: group 1 (GAcetyl) (n=9) receiving intravenous N-acetylcysteine (Zambon, Italy), 300mg/kg[19], and group 2 (GIsot) (n=9) receiving an isotonic saline solution at the same volume. After 30 minutes, a laparotomy was performed, followed by right nephrectomy and left renal artery clamping for 45 minutes using a non-traumatic vascular clamp. After unclamping the renal artery, the venocaths from vein and artery were removed and the abdominal and cervical wall was sutured using non-absorbable thread on a single plane. The isoflurane was interrupted and the animals remained with the tracheal tube and 0.3 FIO_2 until complete recovery from anesthesia. For postoperative analgesia, the surgical wound was infiltrated with 0.25% bupivacaine. The rats were sent to a shelter and kept in a warm environment with water and food. After two

days, the animals were anesthetized again with isoflurane and after performing left nephrectomy and collecting 1 mL of blood for serum creatinine determination, they were sacrificed with an overdose of intra-cardiac sodium pentobarbital. The dry chemistry method was utilized for serum creatinine determination. The kidneys were stored in a DuBoscq Brasil solution (formaldehyde, 6 mL, acetic acid, 15 mL, absolute alcohol, 12 mL and picric acid, 8 mL) for up to 36 hours and, subsequently, in alcohol 70%. Histological analysis was conducted by a single pathologist, who did not have knowledge which group the animals were from, observing the following parameters: tubular degeneration, regeneration and necrosis, and inflammation. The injuries were graded as absent (grade zero), mild (grade I), moderate (grade II), and intense (grade III).

For statistical analysis, the Student *t* test was used for the categorical variables and the Mann-Whitney test for the non-categorical variables. A value of $p<0.05$ was adapted to statistical significance.

Results

Mean and standard deviation for weight (GAcetyl = 450g ± 51.5 and GIsot = 426g ± 59.7) (p = 0.409), for basal creatinine (GAcetyl = 0.27 mg/dL ± 0.11 and GIsot = 0.32 mg/dL ± 0.07) (p = 0.317), for creatinine 48 hours after ischemia/reperfusion (GAcetyl = 2.60 mg/dL ± 2.21 and GIsot = 4.70 mg/dL ± 2.12), for the creatinine delta (GAcetyl = 2.33 mg/dL ± 2.21 and GIsot = 4.38 mg/dL ± 2.13) (p = 0.074) (Figure 1), for the amount of isotonic saline administered to animals (GAcetyl = 5.46 mL ± 0.53 and GIsot = 5.21 mL ± 0.58) (p = 0.382), for rectal temperature during the experiment (GAcetyl = 36.4ºC ±1.02 and GIsot = 37.0ºC ± 0.21) (p = 0.101), for arterial oxygen saturation (GAcetyl = 97% ± 2.1 and GIsot = 98% ± 0.32) (p = 0.271), for ET_{CO2} (GAcetyl = 25 mmHg ± 2.1 and GIsot = 28 mmHg ± 3.9) (p = 0.051), for the expired fraction of isoflurane (GAcetyl = 1.9% ± 0.2 and GIsot = 1.9% ± 0.2) (p = 0.424), MAP1 (GAcetyl = 85 mmHg ± 11 and GIsot = 88 mmHg ± 11) (p = 0.592) and MAP2 (GAcetyl = 80 mmHg ± 16 and GIsot = 85 mmHg ± 13) (p = 0.491) were similar in both groups.

Analysis of the right kidneys did not show histological changes that would significantly differentiate the two groups. However, for the kidneys that suffered ischemia, tubular necrosis was not observed in two GAcetyl kidneys but it was observed in grade II in GAcetyl (4 kidneys) and GIsot (3 kidneys) and grade III in GAcetyl (2 kidneys) and GIsot (5 kidneys) (p = 0.18). Only one GAcetyl kidney did not present tubular degeneration, but there was this change in grade I in GAcetyl (4 kidneys) and GIsot (3 kidneys), grade II in GAcetyl (one kidney) and GIsot (5 kidneys) and grade III only in GAcetyl (3 kidneys) (p = 0.12). No tubular regeneration was observed in four GAcetyl kidneys and in one GIsot kidney, but there was grade I in GAcetyl (2 kidneys) and GIsot (one kidney), grade II in GAcetyl (one kidney) and GIsot (4 kidneys) and grade III in GAcetyl (one kidney) and GIsot (2 kidneys) (p = 0.23). Tubular inflammation was not observed in three GAcetyl kidneys and one GIsot kidney, but it appeared in grade I in GAcetyl (4 kidneys) and GIsot (7 kidneys) and grade II only in GAcetyl (one kidney) (p = 0.24) (Table 1, Figure 2, A, B, C, and D).

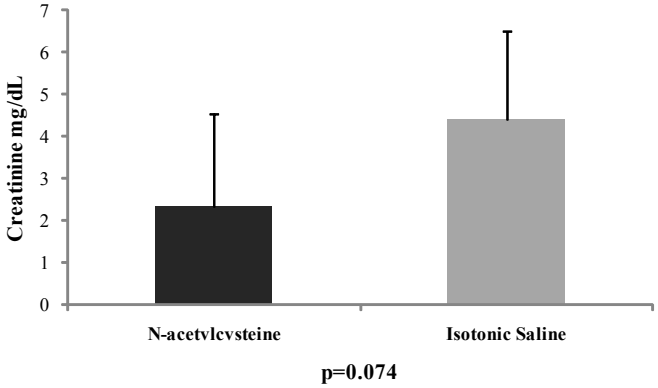

FIGURE 1 - Mean ± standard deviation of variation of serum creatinine (mg/dL) between two time points from administration of acetylcysteine early after anesthesia induction: before ischemia and 48 hours after reperfusion.

TABLE 1 – Number of rat kidneys with histological changes in GAcetyl (group acetylcysteine) and GIsot (group isotonic saline solution) after ischemia/reperfusion. Grade zero – lack of change, I – mild change, II – moderate, III – intense.

Grading of Injuries	Groups (n=9)	Tubular Degeneration	Tubular Necrosis	Tubular Regeneration	Tubular Inflammation
Grade zero	GAcetyl	1	2	4	3
	GIsot	-	-	1	1
Grade I	GAcetyl	4	-	2	4
	GIsot	3	-	1	7
Grade II	GAcetyl	1	4	1	1
	GIsot	5	3	4	-
Grade III	GAcetyl	3	2	1	-
	GIsot	-	5	2	-
p value		0.12	0.18	0.23	0.24

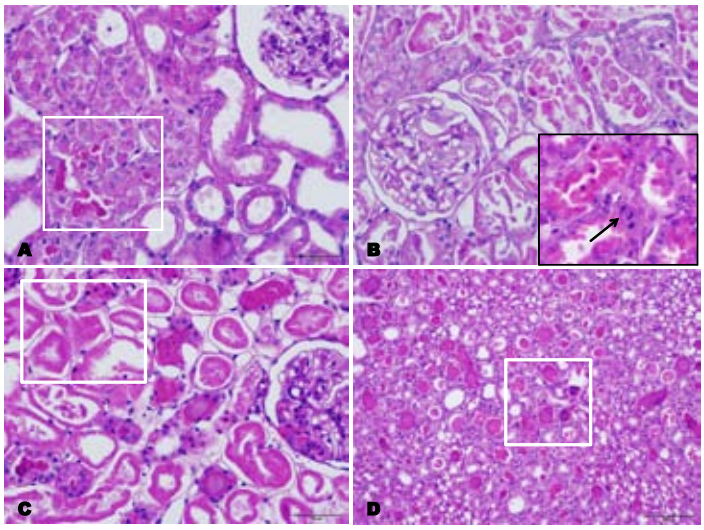

FIGURE 2 – A. Left kidney with tubular necrosis (grade 1, group acetylcysteine): few anucleated (necrotic) tubular eosinophilic cell (HE 400x). **B.** Left kidney with tubular necrosis grade 2 commitment: ischemic tubular necrosis, with early regeneration of tubular cells evidenced by mitotic figures (arrow - added detail) (group acetylcysteine) (HE 400x). **C.** Left kidney with grade 3 necrosis. Most tubules with eosinophilic cells in karyolysis (detail), some still with pyknotic nuclei (group isotonic saline) (HE 400x). **D.** Ischemic left kidney with tubular hyalin casts in the medullary some with focal areas of calcification in detail in blue (group isotonic saline) (HE 200x).

Discussion

This study evaluated the protective role of N-acetylcysteine on a model of kidney ischemia/reperfusion injury similar to what occurs in clinical practice. The N-acetylcysteine was administered after anesthetic induction, mimetizing situations in which there is no chance for previous administrations.

The rectal temperature of animals in this study shows results compatible with mild hypothermia (36.4ºC to 37.0ºC). In clinical practice, the anesthetized patient is expected to have these temperature levels. Profound hypothermia was avoided, which would be a protective factor for the ischemia suffered by the kidneys, and hyperthermia, which would help worsen the kidney injury, changing ATP availability and promoting an increase in oxygen's free radicals[21].

In this study, only the GAcetyl presented animals free of tubular necrosis, and most of the kidneys in these animals presented moderate necrosis of the tubule, whereas in the isotonic saline group most of the kidneys that presented necrosis revealed intense degree. With regard to tubular degeneration, only one kidney from the GAcetyl was without injury, and most of the kidneys in this group had a mild renal tubule injury. Statistical significance probably was not achieved due to the sample number for each group. The same occurred with the variation in creatinine serum levels, which also proved smaller in the GAcetyl - the difference between pre- and post-ischemia serum creatinine is less in the GAcetyl, suggesting some protection.

Nitescu *et al.*[8] did not evidence any mitigation of the kidney injury induced by ischemia/reperfusion in rats anesthetized with thiolbutylbarbital. The dose of N-acetylcysteine was 200mg/kg intraperitoneally, administered 24 and 12 hours before the experiment, following by a 150mg/kg bolus intravenously and 43mg/kg/h in continuous infusion. The time for ischemia was 40 minutes, similar to that used in this study, 45 minutes. The authors postulated that a reduction in glomerular filtration rate found was a result of the loss in tubular cell polarity and the extravasation of tubular fluid, with the development of cylinders, determining tubular obstruction, which definitely would not be inhibited by administration of N-acetylcysteine. In another experiment, the same authors demonstrated, in rats anesthetized with xylazine and ketamine, greater protection against kidney injury induced by ischemia/reperfusion, protection evidenced by lower levels of serum creatinine when compared to isotonic saline. The dose of N-acetylcysteine used was 200mg/kg intraperitoneally, 24, 12, and two hours before the procedure, and 200mg/kg per day, also intraperitoneally, for three days, with an ischemia time of 40

minutes[9].

The animals in the present experiment were anesthetized with isoflurane, an anesthetic that has major influence on organ injuries through pre- and post-conditioning. The mechanism known as ischemic preconditioning (IPC) is an endogenous and fundamental protection against tissue injury[22], observed in many animal species. It was much studied in the heart and well-characterized in this organ, but there is evidence that IPC exists for the kidney[23]. With IPC, brief episodes of ischemia in the heart, occurring before the longest interruption of circulation, protect against organ dysfunction and necrosis[24]. In such a process, the role of the potassium channel, K_{ATP}, is fundamental in the mitochondria, which is sensitive to adenosine triphosphate. This channel opening is critical for the beneficial effects in IPC, cardioprotectors. From an anesthesia perspective, it is important to underscore that several drugs mimetize the IPC phenomenon, which becomes "Pharmacological Preconditioning". Among others, opioid receptor agonists and volatile anesthetics are in this category[25,26].

A study by Zaugg *et al*.[27] demonstrated that volatile anesthetics mimetize cardiac preconditioning by initiating the activation of K_{ATP} mitochondrial channels by multiplying signaling pathways. The anesthetics were grouped in accordance with their potential effect or inhibition of protective activity on isolated cardiac myocytes in adult rats. Thus, isoflurane would increase protection. Research on ischemia/reperfusion injury in rat kidneys in the presence of IPC demonstrated beneficial effects on the organ when the animals were anesthetized with isoflurane associated with remifentanyl[28].

Conclusions

After renal ischemia and reperfusion, rats anesthetized with isoflurane that were given N-acetylcysteine following anesthetic induction had less variation in serum creatinine 48 hours after reperfusion and, although presenting tubular injuries, these were milder than those in the group that did not receive this treatment.

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Received: November 18, 2011
Review: January 14, 2011
Accepted: February 20, 2012
Conflict of interest: none
Financial source: none

[1]Research performed at Experimental Laboratory of Anesthesiology, Botucatu School of Medicine (BSM), Sao Paulo State University (UNESP), Brazil.