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RESEARCH ARTICLE

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# Repeated remote ischemic preconditioning and isoflurane anesthesia in an experimental model of renal ischemia-reperfusion injury

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## Abstract

**Background:** In animal studies, remote ischemic preconditioning (RIPC) and anesthetic preconditioning are successful in reducing renal ischemia reperfusion injury (IRI), however the protective effect of RIPC may be improved by repeating the RIPC stimulus.

**Methods:** Sprague-Dawley rats underwent unilateral nephrectomy followed by 30 min of renal pedicle clamping. Animals were allocated into six groups: sham, control (IRI), ReplISO (daily isoflurane anesthesia), RIPC (single dose isoflurane anesthesia and single dose RIPC), ReplISO + RIPC (7-day isoflurane anesthesia and single dose RIPC) and ReplISO + RepRIPC (7-day isoflurane anesthesia with 7-day RIPC). RIPC was applied by 3x5 min of cuff inflation on both thighs. Serum creatinine and urea levels were measured and histology was obtained at day two.

**Results:** ReplISO diminished renal IRI, as reflected by a significant reduction in serum creatinine levels as compared to the control group,  $170 \pm 74$  resp.  $107 \pm 29$   $\mu\text{mol/L}$ . The other preconditioning protocols showed similar reduction in serum creatinine levels as compared to the control group. No significant differences were observed between the different preconditioning protocols. For urea levels, only ReplISO + RIPC resulted in significantly lower levels as compared to the control group,  $14 \pm 4$  resp.  $22 \pm 7$   $\text{mmol/L}$  ( $p = 0.010$ ). In the preconditioning groups only ReplISO showed less histological damage as compared to controls  $1.73 \pm 1.19$  resp.  $2.91 \pm 1.22$  ( $p = 0.032$ ).

**Conclusions:** In this study no additional protective effect of repeated ischemic preconditioning was observed as compared to single dose RIPC. Repeated administration of isoflurane provided stronger protection against renal IRI as compared to single dose isoflurane.

**Keywords:** Anesthetic preconditioning, Animal experiment, Ischemia reperfusion injury, Kidney, Repeated remote ischemic preconditioning

## Background

Ischemia reperfusion injury (IRI) is tissue damage caused by the restoration of blood flow after a period of deprived circulation of that tissue [1]. The deficit of oxygen and nutrients during the ischemic phase creates a condition in which the return of blood flow induces oxidative stress, inflammation and results in apoptosis of the cell [2].

This may lead to tissue damage and loss of organ function [3]. The kidney is an organ especially vulnerable to IRI, due to its high-energy demand and delicate micro-circulation. IRI of the kidney is a significant clinical problem in shock, renal transplantation and major cardiac or vascular surgery [4]. A promising method to diminish IRI was first described in 1986 by Murry [5], he discovered that short harmless periods of ischemia can protect the heart against a prolonged ischemic period; this phenomenon is called ischemic preconditioning (IPC). It was later described that the interruption of blood flow to

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an organ different than the target organ could also have a protective effect on IRI. This phenomenon is known as remote ischemic preconditioning (RIPC) [6]. Although the exact mechanism of RIPC is unknown, prevention of apoptosis by closure of the mitochondrial permeability transition pores (mPTP), seems to play a pivotal role [3]. A limb is often used as the remote organ for the application of the RIPC stimulus as the blood flow can safely and easily be obstructed by insufflation of a blood pressure cuff around an arm or leg. Experimental studies have shown that RIPC does not only protect against IRI in the heart, but also in other organs, including the kidney [7].

Not only a distant ischemic impulse can cause renal protection from IRI, some anesthetics also protect the kidney against IRI. In myocardial and renal animal studies, [8] anesthetics have shown to reduce IRI in a similar signaling cascade as RIPC, known as anesthetic preconditioning (APC). Volatile anesthetics have extensively been tested for their APC effectiveness in cardiac studies: isoflurane, sevoflurane, desflurane [9, 10], halothane [11] and ether derived anesthetics [12] have proven clinical and preclinical cardioprotective effects. Experiments with intravenous anesthetics, propofol, barbitarates and ketamine [13–15] show no protective effect and have been demonstrated to inhibit mKATP channels which is an indication these anesthetics might diminish the protective effect of APC or RIPC [16]. The effects of multiple periods of anesthetics on IRI are unknown.

In general, animal studies show that RIPC is effective in reducing renal IRI [17]; however, human studies show disappointing results, with a small or non-significant protective effect [18, 19]. Cumulating evidence exists that in cardiac IRI models, repeating the RIPC stimulus over a period of multiple days, repeated RIPC (RepRIPC), could be more effective as compared to single dose RIPC

[13, 20, 21]. It is unclear if this holds true for renal IRI. In this study we test whether the null-hypothesis could be rejected that single dose and repeated RIPC are equally effective in an experimental model of renal IRI.

## Methods

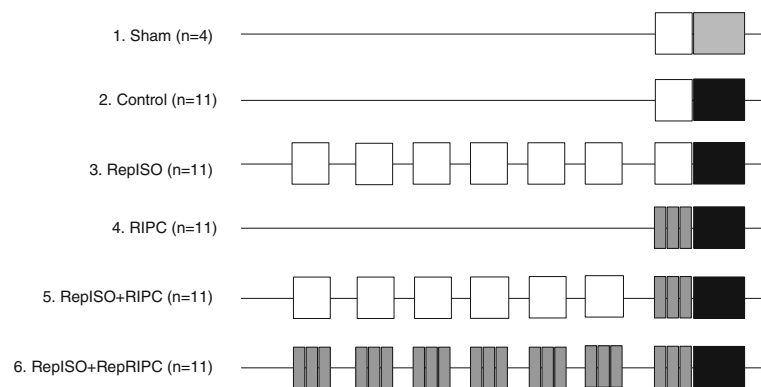
The Committee for Animal Experiments of the Radboud Medical Center, Nijmegen approved all procedures (registration number 20149), and the experiment was conducted according to the ARRIVE criteria. 59 male Sprague–Dawley rats (Harlan Laboratories, Eystrup, Germany) were brought into the facility two weeks before the start of the experiment to acclimatize. Rats from different groups were housed randomly in the same room and under standard specific pathogen-free housing conditions. The environmental temperature was regulated at 22 °C, with a relative humidity of 45% and a 12/12h day/night cycle. At the start of the experiment the animals weight was  $311 \pm 21$ g, at the age of 10 weeks.

## Blinding

Group assignment of each rat was done by computer-generated randomization. The surgeon, caregivers and the analysts performing creatinine, urea and histology measurements were blinded for group assignment of the animals.

## Study design

All animals were anesthetized using isoflurane for the same period of time and all animals underwent right nephrectomy. Animals were randomly divided in six groups (Fig. 1): Group 1 and 2 underwent no preconditioning. The sham group ( $n = 4$ , group 1) underwent a laparotomy, including the resection of the right kidney. The control group ( $n = 11$ , group 2) underwent 30 min of left renal



**Fig. 1** Schematic protocol of the animal groups over the seven days. The open boxes indicate a period of anesthesia alone, gray boxes a period of RIPC and black boxes a period of renal ischemia. Animals were randomly allocated into six groups: sham, control (IRI), ReplISO (daily isoflurane anesthesia), RIPC (single dose isoflurane anesthesia and single dose RIPC), ReplISO + RIPC (7-day isoflurane anesthesia and single dose RIPC) and ReplISO + RepRIPC (7-day isoflurane anesthesia with 7-day RIPC). RIPC was applied by 3×5 min of cuff inflation on both thighs

ischemia (IRI stimulus) during right kidney resection. Groups 3–6 were the experimental, preconditioning groups, all undergoing 30 min of left renal ischemia at the day of surgery and a specific preconditioning stimulus: In group 3; repeated isoflurane (RepISO,  $n = 11$ ), the animals underwent seven days of isoflurane anesthesia for 25 min prior to the day of the operation. In group 4; single RIPC (RIPC,  $n = 11$ ); the animals underwent 3 × 5 min of cuff inflation and 5 min of reperfusion prior to the operation. Cuff inflation was initiated by using human toe pressure cuffs, inflating them simultaneously to 200mmHg on both thighs. RIPC required 25 min of anesthesia as the last 5 min of reperfusion did not require anesthesia. In group 5; repeated isoflurane and a single RIPC stimulus (RepISO + RIPC,  $n = 11$ ), the animals underwent seven days of anesthesia for 25 min prior to the day of the operation. On the day of surgery, during isoflurane anesthesia, 3 × 5 min of cuff inflation on both thighs and 5 min of reperfusion was performed. In group 6; repeated isoflurane and repeated RIPC (RepISO + RepRIPC,  $n = 11$ ), the animals underwent seven days of anesthesia for 25 min together with seven days of 3 × 5 min of cuff inflation on both thighs and 5 min of reperfusion.

### Surgical procedures

All experiments were randomly performed between 8.00 and 16.00h on Mondays and Tuesdays. Preoperative analgesic [Carprofen, 5mg/kg body weight (b.w.)] was administered subcutaneously 30 min prior to surgery. Surgical procedures were conducted using standard aseptic surgical techniques and all microsurgical instruments were sterilized using a dry bead sterilizer (Inothech, Dottikon, Switzerland). Animals were placed on a sterile drape overlying a heating pad to maintain body temperature at 36–38 °C, monitored continuously using a rectal thermometer. Body weights were recorded prior to surgery, prior to blood collection and at the end of the experiment. Anesthesia was induced with 5% isoflurane in pressurized air and maintained at 2.5–3%. Depth of anesthesia was assessed by toe and tail pinch. Preconditioning was done by TM. All operations were done by an experienced microsurgeon (RL), renal ischemia was initiated by blunt dissection of the left renal hilus, and an atraumatic vascular clamp was used to obstruct the venous and arterial blood flow of the kidney. Complete obstruction was confirmed by visualization of the kidney gaining the typical ischemic dark purple color; complete revascularization after removal of the clamp was also visualized before closure of the abdomen. Closure of the abdomen was done by a running suture, securing both ends with a metal clip to prevent opening of the wound by the animal. One day post-operatively, an analgesic (Carprofen, 5 mg/kg b.w.) in 5mL saline was administered subcutaneously.

### Renal function analysis and histology

At baseline, day one and day two blood samples were collected and stored. Blood samples were collected in EDTA tubes and centrifuged for 15 min at 3000g to obtain plasma. Plasma was snap frozen in liquid nitrogen and stored at -80 °C until further use. For the histology, tissue from the remaining kidney was taken two days after surgery and was fixed in 4% paraformaldehyde for at least 48h. For light microscopy of the renal cortex, kidneys were dehydrated and embedded in paraffin. To score renal damage, sections of 4 μm were stained with periodic acid-Schiff. Of each kidney, four sections were taken at different latitudes and scored for damage of the renal cortex and averaged. Damage scoring was performed by a blinded investigator, on a scale from 0 to 4 according to the Jablonski scale [22], with 0: no proximal tubule damaged, and 4: all tubules damaged.

### Statistical and power analysis

Serum creatinine levels were used as the primary outcome measure. Previous experiments have shown that in our model of 30 min renal injury, serum creatinine levels in control animals 48h post-operative are on average 290 μmol/l, with an average standard deviation of 103 μmol/l [23, 24]. We aim to detect a difference in serum creatinine between the RepISO + RepRIPC and all the other experimental groups including the control group of 100 μmol/l. Since there are five comparisons we have adjusted our level of significance for five comparisons, using Bonferonni correction:  $0.05/5 = 0.01$ . In order to achieve a statistical power of at least 80%, we require 11 animals per group. Previous experiments have shown that the standard deviation in sham-operated animals is low (average serum creatinine 48h post-operative =  $46 \pm 8$ ). Therefore 4 animals in the sham group were required. Although the animals were obtained from a different supplier, we estimated that the susceptibility to renal IRI would be similar because the strain, age, sex and weight were identical as in the previously mentioned experiments. All data are presented as mean ± SD unless otherwise specified. The means of the different groups were compared using the Student-t test. The level of statistical significance was set at  $p < 0.05$ . Data were assessed and SPSS 22 and GraphPath 5.03 plotted graphs.

## Results

### Peri-operative complications

Fifty-nine rats were randomly assigned to six different groups. Two rats died during anesthesia. A third animal was excluded at day two of the experiment due to intestinal rotation with obstruction. A fourth rat was excluded because the remaining kidney contained a large tumor, which filled one third of the kidney's volume. The excluded rats belonged to different groups: control,

RepISO, RIPC and RepISO + RepRIPC. The weight of the animals at baseline and the average weight loss at day two in the different groups were not significantly different between the groups.

### Renal function analysis

Serum creatinine (Fig. 2) and serum urea concentrations (Fig. 3) were measured at baseline (ten days before surgery) and on postoperative day one and two. All baseline outcome measures were not significantly different.

In comparison with the control group, all groups showed a significantly lower level of creatinine; control  $170 \pm 74 \mu\text{mol/L}$  vs. sham and experimental group 1 and 3–6 respectively;  $71 \pm 16 \mu\text{mol/L}$  ( $p = 0.023$ ),  $107 \pm 29 \mu\text{mol/L}$  ( $p = 0.022$ ),  $107 \pm 45 \mu\text{mol/L}$  ( $p = 0.032$ ),  $96 \pm 22 \mu\text{mol/L}$  ( $p = 0.007$ ) and  $102 \pm 37 \mu\text{mol/L}$  ( $p = 0.023$ ). For the experimental groups only serum creatinine levels of RepISO on day 1 were significantly higher than sham creatinine levels;  $107 \pm 29 \mu\text{mol/L}$  resp.  $71 \pm 16 \mu\text{mol/L}$  ( $p = 0.039$ ).

On day two the creatinine concentrations were reduced compared with day one and on day two there was no significant difference between sham and the experimental groups. The control animals showed significantly higher creatinine concentrations compared with the experimental groups, RepISO, RepISO + RIPC and RepISO + RepRIPC respectively:  $102 \pm 29 \mu\text{mol/L}$  vs.  $63 \pm 21 \mu\text{mol/L}$  ( $p = 0.036$ ),  $49 \pm 8 \mu\text{mol/L}$  ( $p = 0.006$ ) and  $57 \pm 21 \mu\text{mol/L}$  ( $p = 0.028$ ).

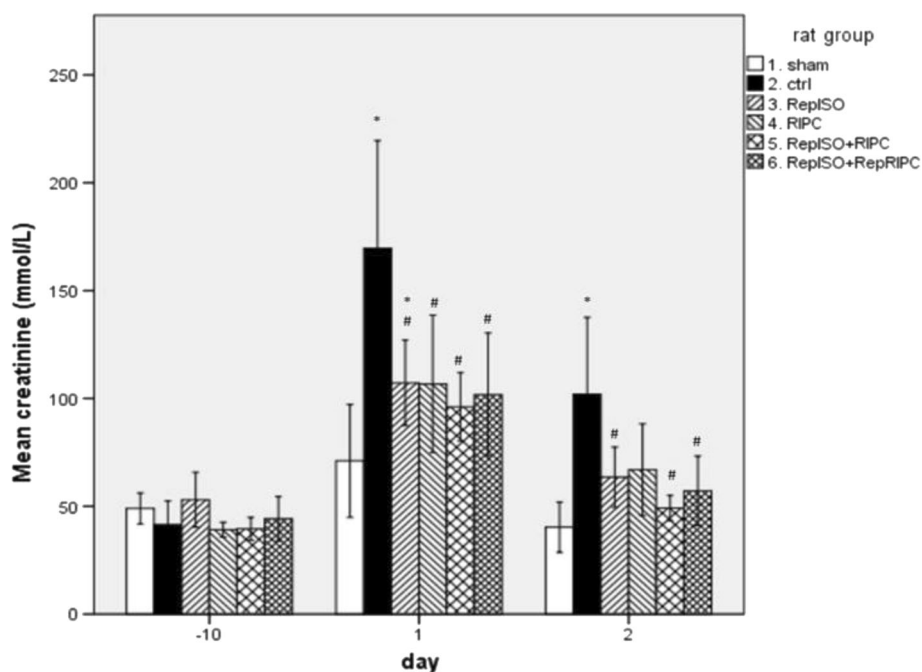
For urea levels, all groups showed significantly higher levels on day one as compared to sham:  $9 \pm 1 \text{ mmol/L}$  vs. control  $22 \pm 7 \text{ mmol/L}$  ( $p = 0.000$ ), vs. RepISO  $18 \pm 5 \text{ mmol/L}$  ( $p = 0.000$ ), vs. RIPC  $17 \pm 8 \text{ mmol/L}$  ( $p = 0.010$ ), vs. RepISO + RIPC  $14 \pm 4 \text{ mmol/L}$  ( $p = 0.002$ ) and vs. RepISO + RepRIPC  $17 \pm 7 \text{ mmol/L}$  ( $p = 0.047$ ). Compared to control operated animals, only serum urea levels in RepISO + RIPC were significantly lower,  $14 \pm 4$  vs.  $22 \pm 7 \text{ mmol/L}$  ( $p = 0.010$ ).

### Histology

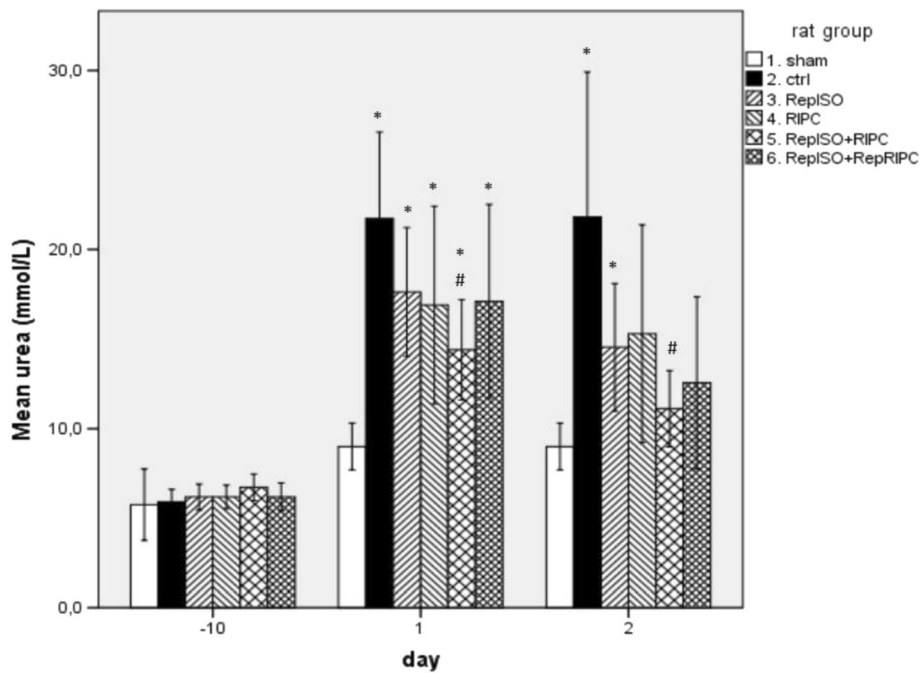
Histology, according to the Jablonski score, showed significantly more renal damage in the control group  $2.91 \pm 1.22$  as compared to sham  $0.75 \pm 0.96$  ( $p = 0.007$ ). In the preconditioning groups only RepISO,  $1.73 \pm 1.19$  ( $p = 0.032$ ), showed significantly less damage as compared to control (Fig. 4).

### Discussion

To our knowledge this is the first experiment of RepRIPC compared to single dose RIPC in an experimental renal IRI model. With regard to the primary hypothesis, we were not able to demonstrate an additive protective effect of a repeated ischemic preconditioning stimulus in this experiment. However the question whether an additive protective effect of RepRIPC does not exist or the unanticipated large reduction in renal IRI by repeated isoflurane blurred the additional protective effects of repeated RIPC, remains unanswered.



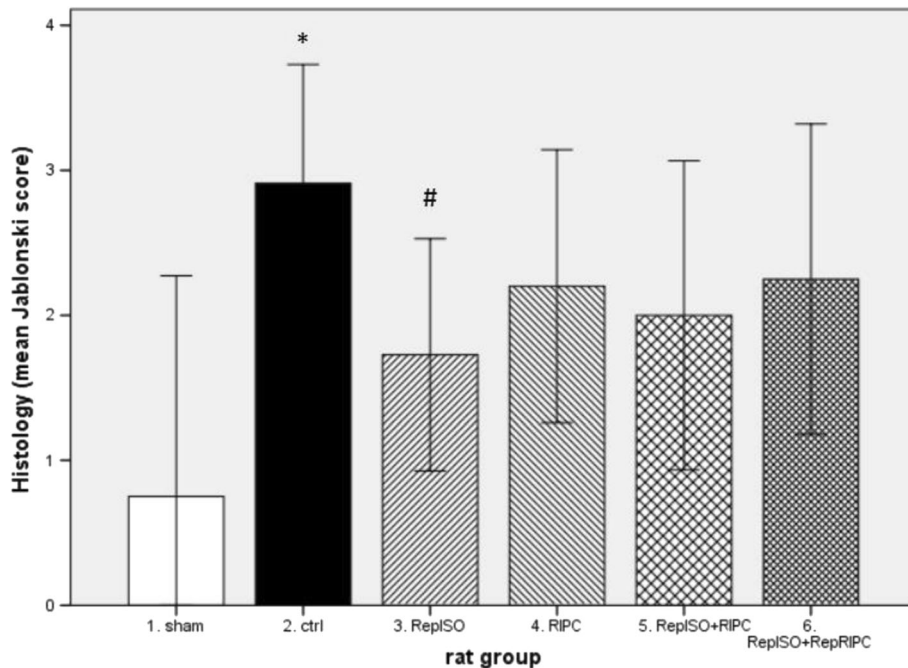
**Fig. 2** Serum creatinine; day -10 (baseline), 1 and 2 postoperative (\* significantly different from sham, # significantly different from control group)



**Fig. 3** Serum urea; day -10 (baseline), 1 and 2 postoperative (\* significantly different from sham, # significantly different from control group)

Results show that all different preconditioning protocols, including RepRIPC, showed a significant reduction in serum creatinine at day one, which was the primary outcome measure. However it is important to note that the observed differences in serum creatinine levels at day one between the different preconditioning protocols

and the control group (single dose APC) were smaller than the difference used for the power calculation (100  $\mu$ mol/L). This indicates that a smaller difference in serum creatinine levels would have been more appropriate to reduce the risk of a type I error. With regard to serum urea levels, only ReplISO + RepRIPC showed a significant reduction as



**Fig. 4** Histology; day 2 postoperative (\* significantly different from sham, # significantly different from control group)



compared to the control group, receiving a single period of isoflurane. Probably the number of animals per group was too small to detect differences in serum urea levels between RepISO and controls. With regard to the histology data, only animals in the RepISO group had lower scores for renal injury as compared to controls. This finding supports the main observation of this study, repeated administration of isoflurane provides stronger protection against renal IRI as compared to single dose isoflurane.

In this study isoflurane was chosen as an anesthetic because it is safe, has little side effects and is widely used in animal studies and in patients. The downside of using isoflurane in this experiment is the protective effect of isoflurane on renal IRI. One previous study [8] showed that single dose isoflurane preconditioning ameliorated IRI of the kidney. In our study we showed that a 7-day repeated isoflurane preconditioning provided significantly more protection against renal IRI as compared to single dose isoflurane in the control group. The smallest number of daily repeated isoflurane preconditioning cycles providing maximum protection remains unknown. To our knowledge, the strong protective effects of repeated isoflurane administrations over multiple days has not been described previously.

Another remarkable observation is that 30 min of pedicle clamping induced less renal injury as compared to our previous experiments [23, 24]. As the amount of renal injury varied between this experiment and previous observations, it would have been better to include more animals to control for this variation in our experimental model. The most likely explanation for the difference with the previous experiments is that our animals were obtained from a different supplier. Despite the fact that we used the same strain, there may have been differences in the genetic makeup leading to a lower susceptibility to renal IRI. This phenomenon is supported by studies showing that different strains of mice have a different susceptibility to cardiac IRI [25, 26]. In this experiment 30 min of IRI was chosen, despite the fact that 45 min is more commonly used in similar experiments [17]. The reason to shorten the IRI period is that the amount of IRI in most animal studies is relatively large as compared to clinical trials [17–19, 21, 27]. In our view the induction of a lower amount of IRI results in a more realistic animal model for the translation into clinical practice.

## Conclusion

IPC has been a promising phenomenon since its discovery in 1986 [5]; however, the vast amount of IRI protection by IPC, shown in animal studies, cannot be translated into clinical trials [17, 19]. Accumulating evidence indicate that RepRIPC is a promising tool to provide a more effective and robust RIPC stimulus. RepRIPC was successfully studied in animal heart models [28, 29], endothelial

dysfunction models in healthy humans [21] coronary artery bypass grafting [30] and after stroke [20]. Nevertheless our results show that it is difficult to establish additional protection of a repeated RIPC stimulus as compared to single dose RIPC in animal studies reducing renal IRI. In future animal studies investigating the mechanisms and/or efficacy of repeated RIPC and APC, the strong protective effects of the repeated administration of (volatile) anesthetics, i.e. isoflurane, should be taken into account.

## Abbreviations

APC: Anesthetic preconditioning; IPC: Ischemic preconditioning; IRI: Ischemia reperfusion injury; mPTP: Mitochondrial permeability transition pores; RepISO: Repeated isoflurane anesthesia; RepRIPC: Repeated remote ischemic preconditioning; RIPC: Remote ischemic preconditioning

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## Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

MW and TM initiated and designed the study. TM, MB, ME and RL performed the experiment, TM and MW analyzed the data. TM wrote the manuscript. HG, MW, and KW revised the manuscript. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interest.

## Consent for publication

Not applicable.

## Ethics approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and practice at which the studies were conducted, in this case the Committee for Animal Experiments of the Radboud Medical Center Nijmegen approved all procedures (registration number 20149), and the experiment was conducted according to the ARRIVE criteria. (<https://www.radboudumc.nl/Research/Organisationofresearch/Departments/cdl/Pages/FacilitiesAndServices.aspx>).

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