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LABORATORY STUDY

Hyperoxia-Induced Protection against Rat's Renal Ischemic Damage: Relation to Oxygen Exposure Time

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Background. Pre-exposure to hyperoxic gas (≥ 95%) has been shown to protect the heart and central nervous system from ischemia-reperfusion injury. In the present study, we investigated whether oxygen pretreatment induces delayed renal protection in rats. The possible role of some renal antioxidant agents was also investigated. *Materials and methods.* Adult male Wistar rats were kept in a hyperoxic (HO) (≥ 95% O_2) environment for 0.5 h, 1 h, 2 h, 3 h, 6 h, and 2 h/day for three consecutive days and 4 h/day for six consecutive days, and control group (IR) animals were kept in the cage with no HO, one day before subjecting their kidney to 40 minutes of ischemia and 24h of reperfusion. Renal function was assessed by comparing plasma creatinine (Cr), blood urea nitrogen (BUN), creatinine clearance (CLCr), and fractional excretion of sodium (FENa%). Histopathological

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injury score was also determined according to the Jablonski method. To examine the antioxidant system induction by hyperoxia, we measured renal catalase and superoxide dismutase activity, and renal glutathione and malondialdehyde content. *Results*. Our data demonstrated that only in 4 h/day HO for six consecutive days, the renal function tests (Cr, CLCr, BUN, and FENa%) and Jablonski histological injury were better than control group ($p < 0.05$). The beneficial effect of oxygen pretreatment in this group was associated with increased renal catalase activity compared with those obtained from control group ($p < 0.05$). *Conclusion.* The present study demonstrates that repeated exposure to hyperoxic (≥ 95% O_2) environment can reduce subsequent rat's renal ischemia-reperfusion damage. Induction of endogenous antioxidant system may partially explain this beneficial effect of hyperoxic preconditioning.

Keywords IR injury, hyperoxia, preconditioning, antioxidant system

INTRODUCTION

Renal ischemia and reperfusion (IR), which is a major cause of acute renal failure (ARF), is a common consequence of surgical intervention in cases of renal transplantation, cardiovascular surgeries, and resuscitation from hypotensive states. Although there were remarkable advances in medical sciences in the half past century, ARF is still a serious clinical problem, and its relatively high mortality and morbidity have not changed considerably.[1,2]

At present, the course of ARF has not been changed remarkably. A beneficial strategy could be finding some ways to prevent postoperative and/or other forms of renal damage.[3] In many organs, a protected state "preconditioning" can be induced, in which the organ becomes tolerant to ischemia. Different stimuli can induce ischemic tolerance, like ischemia itself, heat stress, hypoxia, and oxidative stress.[4,5]

Ischemic preconditioning is a potent mode of organ protection.[4,5] It has been suggested that during brief periods of ischemia and reperfusion, trigger substances like nitric oxide, adenosine, and reactive oxygen species (ROS) are released, and signal transduction cascades of protein kinases activate, which induce cell survival programs.[6–9] Indeed, ischemia and most other stimuli lack potential for clinical use because of associated risks and toxicities. For this reason, safe non-pharmacological stimuli have been sought after, such as hyperoxia, which has been proved to induce tolerance against ischemic damage in rat and mice heart,[9,10] rat brain,[4,11] and rabbit spinal cord.[7] In rat heart, 60 min of hyperoxia (≥ 95% O_2), though in rabbit spinal cord 5 days of 60 min/day hyperoxia, could evoke ischemic protection.

Similar to ischemic preconditioning, hyperoxia has an immediate and a delayed phase of protection with different underlying mechanisms.[8] Some previous studies have postulated that during hyperoxic preconditioning, excess ROS is produced in the body as a result of high oxygen content in the inspired air,[6] activating the cellular antioxidant system. This activation can protect tissues from subsequent ischemia-reperfusion injuries.[12] Hyperoxia, regardless of its underlying mechanisms, is potentially clinically applicable. According to a Gurer et al. study, it seems that there is no early phase of hyperoxia-induced ischemic tolerance in rat renal tissue in exposure times up to six hours[13]; thus, in the present study, we sought to investigate whether hyperoxic (≥ 95% O_2) pretreatment can induce delayed renal protection and to study the impact of the duration of hyperoxic exposure on the possible protection. Also, we tried to determine whether the antioxidant enzymes capacity is affected by repeated hyperoxic preconditioning.

MATERIALS AND METHODS

All the experiments were approved by the Baqiyatallah University of Medical Sciences Ethics Committee for animal use and care.

Animals and Group Assignment

A total of 74 adult male Wistar rats (190–280 g) were used in main groups. All animals were housed at constant temperature (24°C), and a 12 h dark-light cycle was maintained in the animal room. They were allowed free access to food and water before and after surgical intervention and were divided randomly into nine groups:

1. Sham (Normoxy) (n = 5);
2. IR (Normoxy Control) (n = 10);
3. 0.5 h Hyperoxia (HO) + IR (n = 8);
4. 1 h HO + IR (n = 9);
5. 2 h HO + IR (n = 9);
6. 3 h HO + IR (n = 9);
7. 6 h HO + IR (n = 9);
8. 2 h/day HO for three consecutive days + IR (n = 8), "3dHO, 2h/d" group; and
9. 4 h/day HO for six consecutive days + IR (n = 7), "6dHO, 4h/d" group.

Rats in hyperoxic groups were placed into an airtight container (35×25×20 cm) until 24 h before intervention with continuous delivery of oxygen through an inlet. An O_2 analyzer (Lutron DO-5510, Taiwan) was used to monitor the O_2 concentration in the container throughout the exposure time. Rats in sham and IR groups were kept in the same container with continuous delivery of normal air before intervention.

To confirm the rise in arterial O_2 concentration, following the placement in hyperoxic chamber, a gas analyzer (Ciba-Corning 865, Medfield, Massachusetts, USA) was used in another six groups of animals (n = 3) exposed to normoxia and 1/2 h, 1 h, 2 h, 3 h, and 6 h of hyperoxia.

Experimental Protocol

Twenty-four hours after the last session of hyperoxia or normoxia, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Sigma Chemical Co., 50 mg/kg) with concomitant heparin (300 U) injection. Throughout the experiments, their body temperature was kept at 36.5–37.5ºC by placing the rats on a heating pad. After cleaning the abdomen with 10% bovidine iodine, a midline abdominal incision and right nephrectomy were performed. To apply the IR, the left renal artery was clamped with an atraumatic vascular clamp for 40 minutes. IR was confirmed by observing kidney color change and also by measuring its blood flow by a laser Doppler flow meter (Moor instrument MBF3, UK). After removing the clamp, the incision was closed with 2/0 polypropylene sutures. In the sham group, all the procedures were the same except for inducing ischemia. To assess renal function after interventions, each animal was placed in a metabolic cage for 24 h urine collection. The cage had a mechanical mechanism to separate animal stool and food from urine. Twenty-four hours later, blood sample was obtained by cardiac puncture after left nephrectomy. For further biochemical analysis, plasma and urine samples were kept at −24ºC. A piece of left kidney was removed and stored at 10% formaldehyde for histological evaluation, and another piece was immediately washed with normal saline and then frozen in liquid nitrogen for GSH, MDA, SOD, and catalase assays.

Assessment of Renal Function

Urine and plasma samples were processed in an automatic analyzer (Abbott Alycon300) to measure blood urea nitrogen (BUN) and creatinine (Cr). Plasma and urine sodium concentrations were determined using a flame photometer (Seac fp20, Italy). Creatinine clearance (CLCr) (mL/d/kg) was calculated from its standard formula, and fractional excretion of sodium (FENa %) was obtained from the following formula:

$$\text{FENa\%} = [U_{Na} \times VF / P_{Na} \times CLCr] \times 100,$$

when U_{Na} is urine Na concentration, VF is urine volume (mL/d/kg), and P_{Na} is plasma Na concentration.

Antioxidant Enzymes Assessment

Frozen tissue samples were quickly weighed and homogenized 1:10 in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1mM EDTA and proteases inhibitors. The homogenates were then centrifuged at 12000 g for 15 min at 4ºC. The supernatants were taken and used for enzyme, malondialdehyde (MDA), glutathione (GSH), and protein assay.

Catalase and SOD Activities Assay

Catalase (CAT) activity in kidney tissue homogenates was measured by a colorimetric method as described previously by Cohen.[14] The superoxide dismutase (SOD) activity was determined according to the Paoletti method.[15]

GSH and MDA Determination

GSH level was determined by the method of Tietz.[16] MDA production was quantified according to the Ohkawa procedure.[17]

Protein Assay

Total protein concentration was measured by Bradford's method[18] using bovine serum albumin as standard.

Histopathologic Evaluation

Sections were prepared from fixed kidneys using routine histological methods. Thin sections (4–5 μm) were obtained from paraffin embedded kidneys and stained with hematoxylin and eosin. Morphologic evaluation of renal damage was assessed by grading the extent of necrosis of the proximal tubules as described by Jablonski et al.[19] (see Table 1). Light microscopic studies were performed by a pathologist blind to the protocol.

Statistical Analysis

Most data are shown as mean±SEM and were analyzed using one-way analysis of variance (ANOVA) following by Tukey post-hoc test for comparing means from

Table 1
Jablonski grading scale

Score 0	Normal
Score 1	Necrosis of individual cells
Score 2	Necrosis of all cells in adjacent PCT, with survival of surrounding tubules
Score 3	Necrosis confined to distal third of PCT with band(s) of necrosis extending across inner cortex
Score 4	Necrosis of all three segments of PCT

Modified from Jablonski et al. *Transplantation.* 1983;35: 198–204. PCT = proximal convoluted tubule.

different treatment groups. The nonparametric Mann-Whitney U test was used for comparison of Jablonski scores between various groups. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Blood Gas Results

Arterial O_2 concentration in normoxic group (mean ± SEM) was 93.3 ± 2.2 but in hyperoxic groups after 0.5 h, 1 h, 2 h, 3 h, and 6 h was 449 ± 11.6, 433 ± 27.3, 444.7 ± 13.7, 435.7 ± 18.5, and 441.3 ± 10.2, respectively.

Cortical Microcirculatory Blood Flow

There was no difference in cortical blood flow during the intervening procedures in the sham group. In all other groups, there was significant reduction of cortical blood flow during ischemia compared to both pre-ischemic and reperfusion periods ($p < 0.001$). There was no significant difference between pre-ischemic and 24-reperfusion cortical blood flow (data not shown).

Renal Function Tests

The results demonstrated that 40 minutes of left kidney ischemia followed by 24 h reperfusion in this right nephrectomized rat model adversely affected renal function, as reflected by decreased CLCr and increased FENa, plasma Cr, and BUN in the control group, compared with those of the sham group (see Table 2). In hyperoxia preconditioned groups, only six days of 4 h/day oxygen pretreatment resulted in statistically significant improvement in renal function tests compared with control group (see Table 2).

Antioxidant Enzymes Activity

Catalase Activity

Mean renal tissue catalase activity in control group was significantly less than the sham group [29.53 ± 0.65 vs. 40.24 ± 0.74, $p < 0.05$, units/mg protein)]. Only in the "6dHO, 4h/d" group was the catalase activity significantly higher than those of IR group [38.661.58 ± 0.52 (n = 5) vs. 29.53 ± 0.65 (n = 5), $p < 0.05$] and had no statistically differences with sham group (see Figure 1).

SOD Activity

Mean kidney tissue SOD activity in sham group was (51.23 ± 1.58 units/mg protein). This enzyme activity increased in the control group, and only in the "6dHO, 4h/d"group was it significantly lower than the control group (see Figure 1) [53.17 ± 1.66 vs. 62.6 ± 1.3 (units/mg protein), $p < 0.05$] . There were no significant differences between sham and "6dHO, 4h/d" groups in this regard.

GSH and MDA Levels

MDA levels in all groups subjected to IR were not significantly different and were significantly higher than the sham group. GSH level in control group was significantly lower than the sham group. Hyperoxic preconditioning did not changed GSH level in HO groups compared to control group (see Figure 1).

Histology

Marked damage in renal tubular structure was seen after 40 minutes of ischemia followed by 24 h reperfusion. There was heterogeneous loss of brush border, cytoplasmic vacuolization, cellular necrosis, and tubular obstruction. The Joblonski score was the highest in this group (all scores were 4). The sham-operated group did not show any morphological changes (see Figure 2). No significant difference was seen between control and hyperoxic groups except the "6dHO, 4 h/d" group ($p \leq 0.001$). In this group, the Joblonski score in one case was 1, and in others it was 3.This means that necrosis was confined to distal third of proximal convoluted tubule and not extended to all three segments of proximal convoluted tubule, meaning that tubular structure is not seriously damaged.

Table 2
Renal function tests in all studied groups

FENa%	ClCr (ml/day/kg)	BUN (mg/dl)	PCr (mg/dl)	Group
0.069 ± 0.01	2026 ± 194.4	23.40 ± 1.50	0.84 ± 0.024	Sham (n = 5)
3.619 ± 0.49	152.4 ± 73.59	169.1 ± 6.23	3.93 ± 0.27	Control (n = 10)
1.58 ± 0.44	187.6 ± 61.16	162.5 ± 7.39	3.57 ± 0.29	HO (0.5 h)+IR (n = 8)
2.44 ± 0.57	180.4 ± 51.54	152.2 ± 6.76	3.79 ± 0.21	HO (1h)+IR (n = 9)
2.15 ± 0.71	283.3 ± 116.9	144.3 ± 15.02	3.47 ± 0.35	HO (2h)+IR (n = 9)
3.24 ± 0.75	127.5 ± 45.83	165.3 ± 8.29	4.26 ± 0.17	HO (3h)+IR (n = 9)
2.21 ± 0.79	284.6 ± 90.45	119 ± 13.95	3.46 ± 0.50	HO (6h)+IR (n = 9)
2.20 ± 0.45	521.7 ± 202.8	131.8 ±16.63	3.3 ± 0.47	HO (3d/2h/d)+IR (n = 8)
0.40 ± 0.10	962.7 ± 298	81.83 ± 11.81	1.97 ± 0.24	HO (6d/4h/d)+IR (n = 7)

Data are presented as mean±SEM.

Abbreviations: PCr = plasma creatinine, BUN = blood urea nitrogen, CLCr = creatinine clearance, FENa = fractional excretion of sodium.

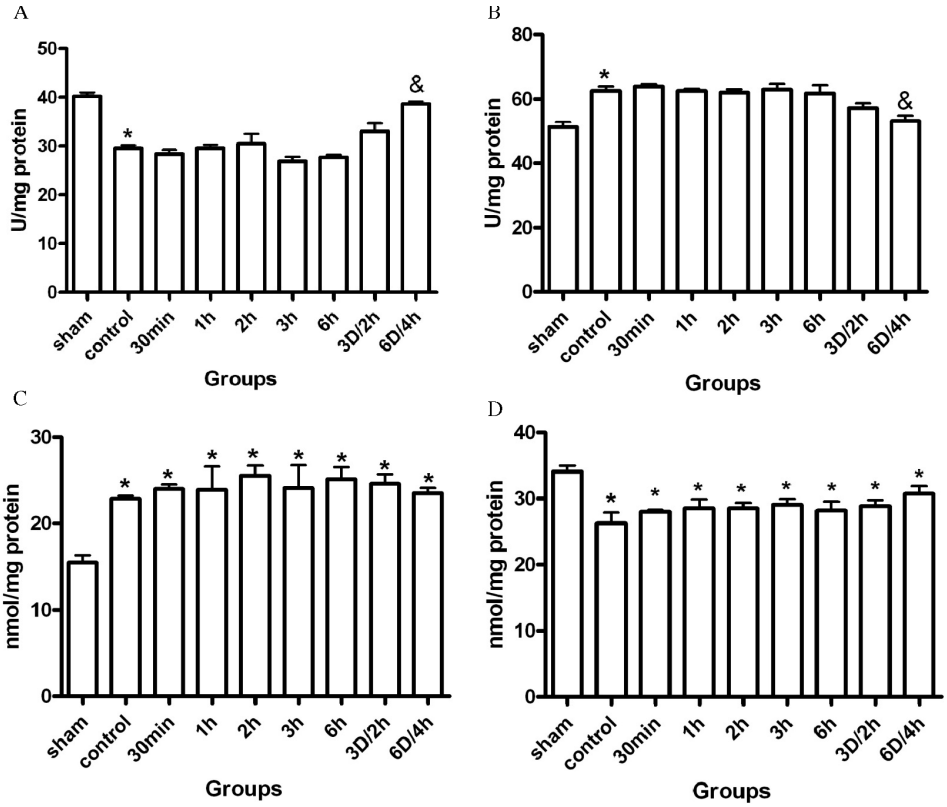

Figure 1. Antioxidant enzymes activity. Activities of (A) catalase (CAT) and (B) superoxide dismutase (SOD); (C) contents of malondialdehyde (MDA) and (D) glutathione level (GSH) in kidney tissues at 24 h after reperfusion in all of our experimental groups. $^{*}p < 0.05$ compared with sham group, $^{\&}p < 0.005$ compared with control group (one-way ANOVA with Tukey post-hoc test).

Cortical Microcirculatory Blood Flow

There was no difference in cortical blood flow during the procedures in sham group. In all other groups, there is significant reduction of cortical blood flow during ischemia compare to both pre-ischemic and reperfusion flow ($p < 0.001$), but no difference was seen between pre-ischemic and 24-h reperfusion cortical blood flow.

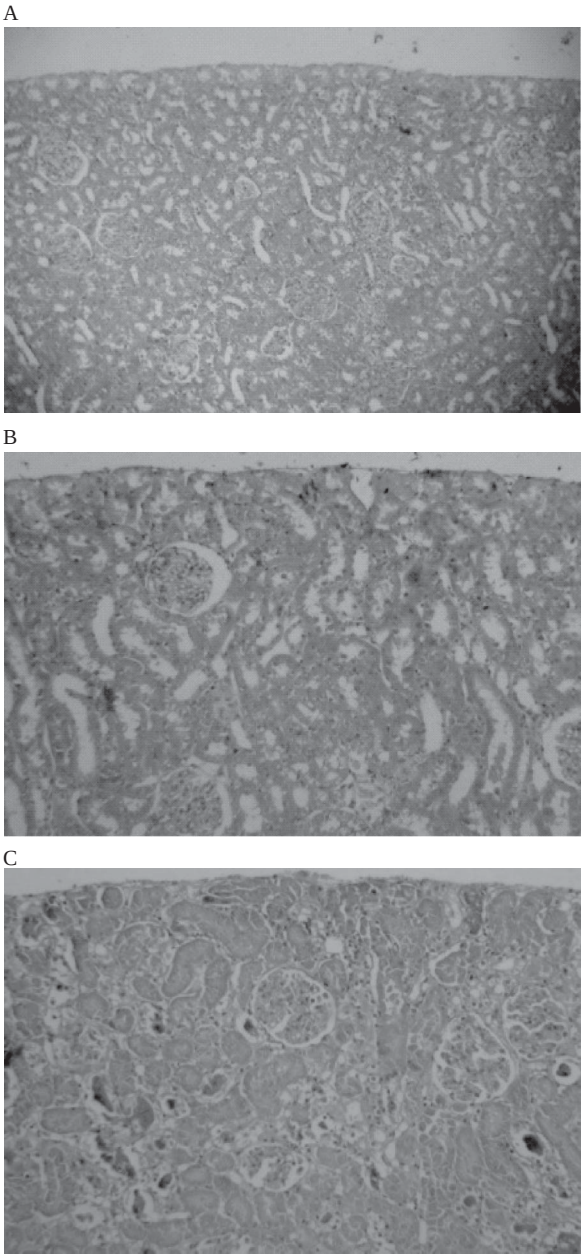

Figure 2. Hystological sections. Light microscopy photographs of kidney specimens (H&E; original magnification ×40). (A) renal cortex of normal kidney; (B) necrotic bands extending from outer medulla to inner cortex, Jablonski score 3; (C) some necrotic tubules in outer cortex, Jablonski score 4.

DISCUSSION

The main finding of the present study was that repeated exposure of rats to hyperoxia (≥ 95% O_2); 6 days of 4h/day; 24 h prior to renal ischemia-reperfusion mimics the beneficial effects of delayed ischemic preconditioning. Renal function tests and histophatological scores were significantly improved in this preconditioned group compared with control group. This beneficial effect of oxygen pretreatment was associated with increased renal catalase activity.

As normobaric hyperoxia could be used in clinical practice more easily than hyperbaric oxygen, we decided to investigate whether "normobaric hyperoxia" can induce delayed phase of renal tolerance against ischemia-reperfusion damage. To our knowledge, besides the few hyperoxic preconditioning investigations performed on rat and mice hearts[10,20] and rat and rabbit central nervous systems,[4,21] there is only one study reported in 2008 about the effect of hyperoxic exposure on inducing rat renal ischemic tolerance.[22] It is not clear in this study that the protective effects of 1 h/day hyperoxic pretreatment for five days are purely due to the late effects of oxygen pretreatment, as the rats were subjected to only in the last day of oxygen exposure. There is also only one study about the early phase of hyperbaric oxygen-induced protection in rat kidney.[13] Thus, in the present study, we have focused purely on the late effects of oxygen pretreatment.

From previous studies, it seems that in tissues like rat heart[6,10] and liver[12] normobaric or hyperbaric oxygen can induce delayed tolerance against ischemic damage in shorter exposure time. For example Esmaili et al. showed in an in vivo model of rat regional heart ischemia that 120 or 180 minutes of nearly pure oxygen pretreatment could induce delayed heart ischemic tolerance,[23] but our study demonstrates that in kidney, like the central nervous system,[4,7] it takes longer to make tissue tolerant against ischemia-reperfusion damage. In 2007, Bigdeli et al. induced delayed rat brain ischemic tolerance after 24 h or 6 days of 4 h/day hyperoxia pretreatment, and Dong et al. induced delayed rat spinal cord ischemic tolerance after 5 days of 1h/day hyperoxia pretreatment.[7]

The underlying mechanisms of hyperoxic and hyperbaric preconditioning are not yet fully understood. It seems that subjecting rats to hyperoxia induces the generation of reactive oxygen species (ROS); therefore, a systemic low-grade oxidative stress is initiated. ROS appear to be the second messenger for induction of antioxidant enzymes.[24] It has been reported that ROS can induce expression of catalase gene[25] and through the activation of NF-kB regulate MnSOD expression.[26] Nie et al. suggested that after hyperbaric preconditioning (5D/1h), the upregulation of antioxidant enzyme activities such as catalase and SOD plays an important role in the induction of tolerance against spinal cord ischemia in rabbits.[27] We saw a decrease in renal catalase activity following IR similar to other published

data, which demonstrates a decrease in catalase activity as well as a reduction of its gene expression after IR.[14,28] The mechanism of reduced catalase activity seems not to be simply the depletion of antioxidant pool,[28] as ROS produced during IR are considered molecules that interact with physiological signal transduction. The general response to hypoxia is to lower gene expression and the activity of antioxidant enzymes such as catalase.[29] Six days of 4 h/day hyperoxic exposure before ischemia increased renal catalase activity probably because ROS generated through a preconditioning period has increased the induction of catalase gene.[24]

In our study, there was a decrease in renal GSH following renal IR, as reported by other studies.[30] This decrease in GSH level could be explained by its consumption for scavenging free radicals and maintaining the redox state of the cell during IR.[31] In the present study, by 6 days of 4 h/day oxygen pretreatment before IR, there was only a non-significant increase in renal GSH level. Similarly Nie et al. showed no significant differences in spinal cord GSH level in hyperbaric preconditioned rabbits 24 after reperfusion.[27]

MDA content is an index of lipid peroxidation. An increased MDA level following IR was seen in our study as in various models of kidney IR injury,[14,28] and after 6 days of 4 h/day hyperoxic preconditioning, there was no change in MDA level in compare with control group. Nearly normal SOD activity in this HO-pretreated group, despite increased renal catalase activity compared to the control group, leads to the reduction of renal H_2O_2 production. However, H_2O_2 diffuses rapidly across biological membranes,[32] and its local injurious effects may be limited in this way. This may explain non-significant change in renal MDA level in this HO-pretreated group compared to control group.

In summary, the present study demonstrates that repeated exposure to hyperoxic ($\geq$ 95% O_2) environment can reduce rat's renal ischemia-reperfusion damage. The induction of endogenous antioxidant systems may partially explain this beneficial effect of hyperoxic preconditioning. Further studies are needed to determine the exact mechanisms of delayed ischemic tolerance induced by repeated oxygen exposure.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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