Preservation of ischemia and isoflurane-induced preconditioning after brain death in the rabbit heart

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Running head: Myocardial preconditioning following brain death.

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ABSTRACT

We sought to determine whether brain death-induced catecholamine release preconditions the heart, and if not, whether it precludes further protection by repetitive ischemia or isoflurane. Anesthetized rabbits underwent 30 min of coronary occlusion and 4 hours of reperfusion. The effect on infarct size of either no intervention (controls), ischemic preconditioning (IPC), or isoflurane inhalation (Iso), was evaluated with or without previous brain death (BD), induced by subdural balloon inflation. Plasma catecholamine levels were measured at several time points.

Although it dramatically increase plasma catecholamine levels, brain death failed to reduce infarct size that averaged 0.49±0.34 without, versus 0.45±0.27 g with BD. IPC and Iso, alone as well as following brain death, significantly reduced infarct size, that averaged 0.11±0.04*, 0.21±0.15*, 0.10±0.09* and 0.22±0.10 g* in IPC, Iso, BD+IPC and BD+Iso groups, respectively (mean ± SD, *p<0.05 vs controls).

Brain death-induced catecholamines “storm” does not precondition the rabbit heart that however retains the ability to be protected by repetition of brief ischemia or isoflurane inhalation.
**Introduction**

Pharmacological stimulation of α-adrenergic receptors, either with exogenous norepinephrine or via release of endogenous catecholamines has been shown to trigger preconditioning in some preparations (3,4,16,25). These experimental observations might be of major clinical importance in the settings of brain death, which is accompanied by an acute and dramatic sympathetic stress (5,22). Hearts of brain dead patients may be further used as donor organs, and thus submitted to prolonged ischemia followed by reperfusion before transplantation. Despite protection of the cardiac graft using hypothermia and preservation solutions, the myocardium may be damaged, sometimes irreversibly.

It is therefore of major clinical importance to determine whether brain death-induced catecholamine release may protect the heart, and if not, whether it retain the ability to be preconditioned, in order to improve overall cardiac graft protection.

The general objective of the present study was to investigate whether brain death may precondition the rabbit heart, and, if not, whether preconditioning can still be induced using the clinically available mitochondrial K$_{ATP}^+$ activator isoflurane.


**Materials and Methods**

All animals were treated in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication N° 85-23, revised 1996).

**Surgical preparation**

New Zealand white rabbits of either sex, weighing 2.5 ± 0.5 kg, were premedicated with an intra-muscular injection of xylazine (5 mg.kg⁻¹) and anesthetized with ketamine (50 mg.kg⁻¹, intramuscularly), as previously described (10). Anesthesia was maintained by a continuous infusion of thiopental (30 mg.kg⁻¹.h⁻¹, iv). After tracheotomy, animals were mechanically ventilated (Servo ventilator 900B, Siemens-Elema, Solna, Sweden) using a tidal volume of 15 ml.kg⁻¹, a frequency of 35 breaths.min⁻¹ and an oxygen fraction of 50%. When needed, adjustments were made to keep the end-tidal carbon dioxide within the physiologic range. End tidal gas concentrations were measured continuously using a gas analyzer (Capnomac Ultima, Datex, Helsinki, Finland). Limb lead II of the ECG was recorded throughout the experiment. Core temperature was maintained between 38 and 39°C by means of a heating system incorporated into the operating table. Systemic blood pressure was monitored by use of a Gould pressure transducer connected to a fluid filled catheter inserted in the left femoral artery. Infusion of fluids (hetastarch: 5 ml.kg⁻¹.h⁻¹) and drugs was performed via a catheter positioned into an ear vein.

After an intravenous bolus administration of fentanyl (25 µg), the heart was exposed via a left thoracotomy and suspended in a pericardial cradle. A 4/0 suture was passed around the first large marginal branch of the circumflex artery to further perform coronary occlusion. In all
animals, a craniotomy was performed approximately 5 mm from the sagittal suture, at the fusion of the parieto-occipital plates. An electroencephalogram [EEG] (Reega Minihuit-TR, Alvar Electronic, Montreuil-Paris, France) was recorded using electrode needles positioned on the two parieto-occipital skull areas. After the surgical preparation, 30 minutes of stabilization were allowed.

**Induction of brain death**

In brain death groups, a 10 Ch Foley catheter was introduced into the subdural space. Brain death [BD] was induced by injection of 5 ml of a normal saline solution into the catheter balloon over 10 seconds. Disappearance of EEG waves, occurrence of a bilateral fixed mydriasis and disappearance of spontaneous respiration ascertained brain death. After injection, the catheter was kept inflated until the end of the experience.

**Experimental design**

The present study aimed at determining: (1) whether BD may protect the heart against infarction, and (2) whether preconditioning may still be induced in brain death rabbits using either ischemia or halogenated anesthetics as a trigger.

In all groups, the coronary artery was occluded for 30 minutes. Myocardial ischemia was confirmed by the appearance of a regional cyanosis, akinesia or dyskinesia and a marked ST segment elevation in the ECG. After 30 minutes, the snare was released and reperfusion was allowed for a period of 4 hours. Reperfusion was visually confirmed by the disappearance of epicardial cyanosis.

Before the sustained 30 minute coronary artery occlusion, three groups of rabbits underwent BD, either alone (BD group), or associated with two episodes of 5 minute ischemia and 10 minutes of reperfusion (BD+IPC group), or with one episode of 15 minutes isoflurane
inhalation (1 MAC = 2% end tidal concentration) followed by a 15 minute washout period (BD+Iso group) (8) (Figure 1). In the three remaining groups, BD was not performed before the sustained occlusion; rabbits underwent either no intervention (C group), two episodes of 5 minutes ischemia and 10 minutes of reperfusion (IPC group), or one episode of 15 minute of isoflurane inhalation (1 MAC = 2% end tidal concentration) followed by a 15 minute washout (Iso group), as previously described (9). For each animal receiving isoflurane, end tidal concentration was less than 0.1% at the end of the washout period.

Area at risk and infarct size measurement

At the end of the 4 hour reperfusion, the coronary artery was briefly reoccluded. Uniperse blue (Ciba-Geigy, Hawthorne, NY) was injected via the ear vein catheter to delineate the area at risk. Euthanasia was then induced, under deep anesthesia, by an intravenous injection of 4 mEq of KCl. The heart was excised and, after removal of the right ventricle, cut into five or six two millimeter thick transverse slices. Each slice was weighed. Its basal surface was photographed. Each slice was then incubated for 20 minutes in triphenyl tetrazolium chloride (at 37°C) and re-photographed for measurement of infarct size. Extent of left ventricle area [LV], area at risk [AR] and area of necrosis [AN] were quantified by computerized planimetry and corrected for the weight of tissue slices. Total weights of AR and AN were then calculated and expressed as weight (grams).

Plasma catecholamines

Arterial plasma samples were immediately centrifuged (3000 rotation.min⁻¹, at +4°C, for 10 minutes) and stored at −80°C until measurement. Plasma levels of epinephrine [E] and norepinephrine [NE] were assessed using the Chromsystems kit for high-performance liquid chromatography [HPLC] analysis with electrochemical detection. Normal reference values
Plasma catecholamines were measured at baseline, and at 1 and 5 minutes after BD [respectively baseline, T1 and T5] as well as just before the prolonged coronary occlusion [pre-occlusion].

**Hemodynamics**

Heart rate, systolic and diastolic blood pressure (HR, SBP, DBP) were assessed at baseline, 1 and 5 minutes after BD [respectively baseline, T1 and T5], just before coronary occlusion [pre-occlusion], at the end of coronary occlusion [30’occlusion] and at 1, 2, 3 and 4 hours after reperfusion [respectively R1, R2, R3 and R4].

**Statistical analysis**

Statistical analyses of hemodynamics and plasma catecholamines were performed using two-way analysis of variance with repeated measures on one factor. LV weight and area at risk were analyzed by analysis of variance. Effect of pretreatment on percent of risk zone infarcted was analyzed by one-way analysis of variance followed by post hoc LSD test when appropriate. The difference of infarct size among groups was evaluated by analysis of covariance, with area of necrosis as the dependent variable and area at risk as the covariant. \( p < 0.05 \) was considered statistically significant. Data are expressed as mean ± standard deviation.
Results

Mortality and exclusion

Among the 48 rabbits that were included in this study, 3 were excluded for technical problems during the surgical preparation (one in the IPC group, one in the BD+IPC group and one in the BD+Iso group). Data are therefore presented for 45 rabbits: 8 in each of the control (C), brain death (BD) and isoflurane (Iso) groups; 7 in the ischemic preconditioning (IPC), brain death + ischemic preconditioning (BD+IPC) and brain death + isoflurane (BD+Iso) groups.

Hemodynamics

In the control group (C), heart rate and blood pressure remained stable throughout the experiment (table 1). As expected, brain death resulted in a dramatic increase in systolic arterial pressure and heart rate (table 1). This hemodynamic response was however short-lived since both systolic blood pressure and heart rate returned to near control values at the onset of the prolonged coronary artery occlusion. In the IPC and Iso groups, blood pressure and heart rate did not significantly differ from control throughout the experiment. Both BD+IPC and BD+Iso groups displayed a hyperdynamic response comparable to that of the BD group following brain death induction. During the final reperfusion, systolic blood pressure was consistently lower in the three brain death groups when compared to controls (table 1).

Catecholamines

The hyperdynamic response was simultaneous to a major rise of plasma catecholamine levels in the brain death groups (table 2). One minute after brain death, norepinephrine averaged $8.64\pm5.79^*$, $4.48\pm3.26^*$ and $13.86\pm17.22^*$ nmol.l$^{-1}$, in the BD, BD+IPC and BD+Iso groups.
respectively, significantly different from 0.41±0.15 in the C group. Epinephrine plasma levels averaged 1.82±1.85*, 1.20±1.15* and 2.82±3.27* nmol.l⁻¹, in the BD, BD+IPC and BD+Iso groups respectively, versus 0.34±0.17 in the C group (p<0.01 for all groups) (table 2). At T1 or T5, plasma levels of E and NE were not significantly different among BD, BD+IPC and BD+Iso groups. Plasma catecholamine levels failed to significantly vary in IPC and Iso groups.

**Infarct size**

LV weight and area at risk were comparable among the different groups (table 3 and figure 2). Brain death failed to significantly alter infarct size that averaged 0.45±0.27 g in the BD group versus 0.49±0.34 g in controls (p=ns). This was confirmed when the weight of the infarct size was plotted versus the weight of the area at risk (figure 3). Clearly, all points in the BD group lie close to the control regression line, indicating that for any value of area at risk, BD hearts developed infarct size comparable to controls. As expected, ischemic preconditioning and isoflurane-treated animals developed significantly smaller infarcts than controls: 0.11±0.04 g and 0.21±0.15 g in IPC and Iso groups, respectively (p<0.05 versus C). This infarct size limitation persisted in the BD+IPC and BD+Iso groups, with a mean area of necrosis averaging 0.10±0.09 g and 0.22±0.10g, respectively (p<0.05 versus C)(table 3 and figure 2). These results were confirmed when the weight of the infarct size was plotted versus the weight of the area at risk (Figure 4). As depicted in panel A, data points for the IPC and the BD+IPC groups lie below the control line, indicating that for any size of the risk region, ischemic preconditioning alone (IPC) as well as ischemic preconditioning in brain death animals (BD+IPC) resulted in significantly smaller infarcts than controls. Panel 4B shows a similar result with in isoflurane-treated groups: when performed with or without brain death, isoflurane inhalation significantly decreased infarct size irrespective of the size of the risk
region. There was no correlation between infarct size and plasma levels of catecholamines in the brain death groups (BD, BD+IPC, BD+Iso).
Discussion

In the present study, we demonstrated that brain death alone fails to protect the rabbit heart, yet does not prevent induction of preconditioning using a brief episode of ischemia or pharmacological activation of mitochondrial $K^+_\text{ATP}$ channels.

Several studies suggested that endogenous release of catecholamines before a prolonged ischemic insult can protect the heart. Transient induction of norepinephrine release by tyramine before a prolonged coronary artery occlusion limits infarct size in the rabbit heart (23). Depletion of presynaptic nerve terminals of norepinephrine stores using reserpine prevents ischemic preconditioning (3,24). In addition, exogenous norepinephrine can trigger a protection that is abolished by the alpha1-adrenoreceptor blocker prazosin in both in vivo rabbit or isolated rat hearts (4,13). Although important, these experimental designs do not truly refer to clinical situations.

In contrast, catecholamine release in brain death experimental preparations clearly depicts the clinical scenario preceding cardiac transplantation. Brain death induces a transient and massive catecholamine release, and the donor heart further undergoes prolonged global ischemia before reperfusion at the time of transplantation. Although the above cited studies would suggest that the soon-to-be transplanted heart may be protected following brain death, the question remains unresolved. In the present study, we were unable to demonstrate any beneficial effect of brain death-induced catecholamine release on the heart. Plasma catecholamine release was transient but of major amplitude, like in the clinical situation, and involved both norepinephrine and epinephrine. Absence of a protective effect cannot be due a detrimental effect of the hemodynamic response, since both heart rate and blood pressure returned to near baseline levels at the onset of the sustained coronary artery occlusion, and heart rate and systolic blood pressure are not major determinants of infarct size in this
preparation. One cannot rule out that the ischemia-reperfusion challenge designed in our protocol might have been too severe for a norepinephrine-induced protective effect to be effective, whereas such a putative protection may have been unmasked following prolonged global hypothermic ischemia and reperfusion like occurs in the clinical settings. The apparent discrepancy of our results with studies demonstrating a role for catecholamines in preconditioning is unclear. One must yet mention that, in reserpinized rabbits, Ardell et al. were able to induce ischemic preconditioning using four, but not one, cycles of brief ischemia-reperfusion (1). Haessler et al. failed to prevent ischemic preconditioning with alpha1-adrenoreceptor blockers (11). Sebbag et al. could not protect the dog heart by intracoronary administration of the alpha1-adrenoreceptor agonist methoxamine (21). In contrast, our results are in close agreement with those of de Zeeuw et al. who reported that transient intracerebral hypertension cannot precondition the pig heart despite a major myocardial norepinephrine release, as demonstrated by microdialysis (8). Also, Kirsch et al. recently showed that brain death does not trigger preconditioning in the rabbit (15).

Conversely, some reports established a detrimental influence of catecholamines on the myocardium (17,20). Following an acute increase in intracranial pressure, brain death results in a major neuronal depolarization and catecholamine release that may induce myocardial contractile dysfunction and, in some case, minimal focal necrosis (12,22). Recently, Communal et al. demonstrated that norepinephrine induces apoptosis of cultured adult rat cardiomyocytes via beta1-adrenoreceptors activation (6,7). One may hypothesize that brain death might induce some irreversible damage, e.g. through apoptosis, that may have blunted the putative preconditioning effect of catecholamine release on infarct size limitation. This is however unlikely since myocardial damage possibly induced by catecholamine rarely exceed small foci of necrosis. Finally, it is possible that, although plasma levels of norepinephrine and epinephrine were dramatically increased in our preparation, their concentration within the
myocardium remained beyond a given threshold necessary to trigger preconditioning, as suggested by de Zeeuw et al. (8).

The lack of donor organs currently limits the availability of heart transplantation, and as much as 20% of potential cardiac grafts display myocardial dysfunction. Interestingly, in the present study, hearts from brain death animals could still be protected against further ischemia-reperfusion. These hearts preserved the ability to be protected by ischemic preconditioning with an infarct size reduction similar to that observed in non-brain death preconditioned rabbits. This indirectly suggests that the absence of infarct size limitation following brain death alone is likely not due, like hypothesized above, to the fact that the putative norepinephrine-induced preconditioning was masked by a concurrent catecholamine cardiotoxicity, but rather simply reflects the lack of efficiency of the catecholamine stimulus to precondition the heart in our experimental conditions. Our results are in contradiction with those of Kirsch et al. who reported that ischemic preconditioning cannot be triggered in brain dead rabbits (15). These discrepancies between the two studies may be due to the fact that: 1) TTC determination of infarct size was performed after only 90 minutes of reperfusion in their study (versus 4 hours in the present work), and 2) mostly, Kirsch et al. used one single sequence of 3 minutes ischemia and 3 minutes reperfusion to precondition brain dead rabbit hearts. It is quite possible that, as suggested by Baines et al., a minimal threshold of ischemic insult be required to induce preconditioning in some circumstance (2). Importantly, isoflurane inhalation prior to the sustained ischemic insult, afforded similar protection, indirectly suggesting a role of mitochondrial $K^{+}_{ATP}$ channels activation, as previously shown (14,19). This strongly suggests that the signalling pathways of ischemic preconditioning, and possibly mitochondrial $K^{+}_{ATP}$ channels, retain their functionality following brain death. In other words, the massive sympathetic activation, although it undoubtedly altered respiration rate, ATP production and matrix Ca$^{2+}$ concentration, does not seem to alter the role of mitochondria in
preconditioning. Yet, in our study, the protection afforded by isoflurane might also result, as recently suggested by Miura et al., from an attenuation of cardiac sympathetic nerve injury provided by the ATP-sensitive potassium channel opener, isoflurane (18).

The present observation is of potential major clinical importance since isoflurane inhalation is feasible in the situation of human brain death to further protect the donor organ before cardiac transplantation. This however needs further investigations to be fully determined.
Acknowledgments

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References


FIGURE LEGENDS

Figure 1: Experimental protocol

C = control group, BD = brain death group, IPC = ischemic preconditioning group, Iso = isoflurane group, BD+IPC = brain death + ischemic preconditioning group, BD+Iso = brain death + isoflurane group.

Figure 2: Infarct size expressed as percentage of area at risk for each individual animals

C = control group, BD = brain death group, IPC = ischemic preconditioning group, Iso = isoflurane group, BD+IPC = brain death + ischemic preconditioning group, BD+Iso = brain death + isoflurane group. * = significantly different from control group (p<0.05). Brain death had no effect on infarct size. Ischemic preconditioning and isoflurane preadministration significantly reduced infarct size expressed as percentage of risk area. This protection was preserved after brain death.

Figure 3: Infarct size (necrosis in grams) plotted as a function of area at risk (grams).

C = control group, BD = brain death group. AR = area at risk, AN = area of necrosis. Each point represent one individual experiment. Regression lines for each group are calculated by the least square method. Data points for brain death group lie close to those of the control group, indicating that brain death had no effect on infarct size.

Figure 4: Infarct size (necrosis in grams) plotted as a function of area at risk (grams).

C = control group, IPC = ischemic preconditioning group, Iso = isoflurane-induced preconditioning group, BD+IPC = brain death + ischemic preconditioning group, BD+Iso = brain death + isoflurane-induced preconditioning group. Each point represents one individual animal. Regression lines for each group are calculated by the least square method.
A: Data points for the IPC and the BD+IPC groups lie below the C group regression line, indicating that they developed a smaller infarct for any size of area at risk (p<0.05).
B: Data points for Iso group and BD+Iso group lie below the C group regression line, indicating that they developed a smaller infarct for any size of area at risk (p<0.05).
Table 1: Hemodynamic measurements in different experimental groups

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<tr>
<th></th>
<th>C</th>
<th>IPC</th>
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<th>BD</th>
<th>BD+IPC</th>
<th>BD+Iso</th>
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<td>152±16*</td>
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<td>T 5</td>
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<td>75±10</td>
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C = control, BD = brain death, IPC = ischemic preconditioning, Iso = isoflurane
T1 = 1 min after brain death, T5 = 5 min after brain death
Values are expressed as mean ± SD
* significantly different from C group at the same time (p<0.05)
Table 2: Plasma catecholamines levels in different experimental groups

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<td>0.30±0.16</td>
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<td>Baseline</td>
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<td>0.14±0.14</td>
<td>0.40±0.25</td>
<td>0.17±0.09</td>
<td>0.16±0.09</td>
</tr>
<tr>
<td>Pre-occlusion</td>
<td>0.26±0.16</td>
<td>0.16±0.10</td>
<td>0.09±0.10</td>
<td>0.16±0.12</td>
<td>0.19±0.11</td>
<td>0.12±0.07</td>
</tr>
</tbody>
</table>

C = control, BD = brain death, IPC = ischemic preconditioning, Iso = isoflurane
T1 = 1 min after brain death, T5 = 5 min after brain death
Values are expressed as mean ± SD
* significantly different from control group at the same time (p<0.05)
Table 3: Area at risk, Infarct size and risk zone infarcted:

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>IPC</th>
<th>Iso</th>
<th>BD</th>
<th>BD+IPC</th>
<th>BD+Iso</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV weight (g)</td>
<td>3.09±0.52</td>
<td>2.89±0.37</td>
<td>2.80±0.38</td>
<td>3.21±0.30</td>
<td>3.22±0.45</td>
<td>2.70±0.35</td>
</tr>
<tr>
<td>Area at risk (%LV)</td>
<td>28.8±8.1</td>
<td>27.3±4.7</td>
<td>27.6±6.6</td>
<td>28.2±10.1</td>
<td>29.2±5.9</td>
<td>31.7±10.4</td>
</tr>
<tr>
<td>Area at risk (g)</td>
<td>0.91±0.36</td>
<td>0.79±0.18</td>
<td>0.77±0.17</td>
<td>0.90±0.31</td>
<td>0.94±0.21</td>
<td>0.83±0.21</td>
</tr>
<tr>
<td>Infarct size (%LV)</td>
<td>15.2±8.9</td>
<td>3.8±1.7*</td>
<td>7.3±5.6*</td>
<td>14.3±9.1</td>
<td>3.2±2.8*</td>
<td>8.5±4.6*</td>
</tr>
<tr>
<td>Infarct size (g)</td>
<td>0.49±0.34</td>
<td>0.11±0.04*</td>
<td>0.21±0.15*</td>
<td>0.45±0.27</td>
<td>0.10±0.09*</td>
<td>0.22±0.10*</td>
</tr>
<tr>
<td>Infarcted risk zone (%)</td>
<td>49.2±17.6</td>
<td>14.1±6.8*</td>
<td>24.7±16.5*</td>
<td>47.1±18.2</td>
<td>10.0±6.8*</td>
<td>25.1±8.4*</td>
</tr>
</tbody>
</table>

C = control, BD = brain death, IPC = ischemic preconditioning, Iso = isoflurane, LV = left ventricle. T1 = 1 min after sham or brain death, T5 = 5 min after sham or brain death. Values are expressed as mean ± SD. * significantly different from control-group at the same time (p<0.05).
**Fig 1. Experimental protocol.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>Oclusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>15 min</td>
<td>30 min</td>
<td>4 hr</td>
</tr>
<tr>
<td>IPC</td>
<td>15 min</td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Iso</td>
<td>15 min</td>
<td>Isoflurane 15 min</td>
<td>Washout 15 min</td>
</tr>
<tr>
<td>BD</td>
<td>15 min</td>
<td>Oclusion 30 min</td>
<td>Reperfusion 4 hr</td>
</tr>
<tr>
<td>BD+IPC</td>
<td>Brain Death</td>
<td>15 min</td>
<td>Oclusion 30 min</td>
</tr>
<tr>
<td>BD+Iso</td>
<td>Brain Death</td>
<td>Isoflurane 15 min</td>
<td>Washout 15 min</td>
</tr>
</tbody>
</table>

C = control, BD = brain death, IPC = ischemic preconditioning, Iso = isoflurane-induced preconditioning. Occl = Occlusion, Reperf = Reperfusion.
Infarct size (% of area at risk)

C  IPC  Iso  BD  BD+IPC  BD+Iso
Fig 3.
Fig 4.

A

- C
- IPC
- Iso

AN (g)
AR (g)

B

- BD
- BD+IPC
- BD+Iso

AN (g)
AR (g)