Remote Limb Ischemic Post-conditioning Improves Post-resuscitation Cerebrovascular Circulation and Survival/Neurological Prognoses via in situ and Remote Activation of Akt-eNOS-NO Signaling Pathway

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Post-conditioning and Cardiac Arrest/CPR

• Ischemic pre- and post-conditioning have been shown to protect against ischemia/reperfusion (I/R) injury.
• Ischemic post-conditioning is clinically more practical and can be applied to a number of vital organs after the I/R insult (e.g. myocardial infarction, organ transplantation, etc).
• For global I/R injury such as cardiac arrest and CPR, ischemic post-conditioning during CPR by cyclic interruption of chest compression has been shown to improve cardiac and cerebral recovery.

Segal N, et al. Resuscitation 2012
Experimental paper

Ischemic postconditioning at the initiation of cardiopulmonary resuscitation facilitates functional cardiac and cerebral recovery after prolonged untreated ventricular fibrillation

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\textbf{Standard CPR with ischemic postconditioning (SCPR+PC) protocol}

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15 minutes of untreated VF

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\begin{itemize}
  \item CPR 40sec
  \item Pause 20sec
  \item CPR 20sec
  \item Pause 20sec
  \item CPR 20sec
  \item Pause 20sec
  \item CPR
  \item ROSC
\end{itemize}

---

Aortic pressure tracing (mmHg)

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0.5 mg of Epinephrine

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ROSC

---

\textbf{Fig. 1.} Ischemic postconditioning protocol during standard cardiopulmonary resuscitation. In the SCPR + PC group during the first 3 min of CPR, animals received four 20-s pauses and each pause was followed by 20 s of SCPR. The “stuttering” introduction of reperfusion is called “ischemic postconditioning”. SCPR: standard CPR; VF: ventricular fibrillation; ROSC: return of spontaneous circulation.
Remote Ischemic Post-conditioning

- After return of spontaneous circulation (ROSC), induction of whole-body ischemic post-conditioning by cyclic cessation and resumption of global circulation is technically difficult and unethical.
- Under such circumstances, remote ischemic post-conditioning by cyclic limb arterial occlusion - reopening is more practical and proves to improve survival and neurological prognosis.

Mechanisms of Post-conditioning

• NO generated by eNOS is crucial for vascular function and homeostasis, and plays a crucial role in the protection of ischemic pre- and post-conditioning.
• Nitric oxide synthase (NOS) plays an important role in physiological and pathological events in CNS.
• Endothelial NOS (eNOS) is found in a subset of neurons and in the endothelium of cerebral blood vessels.
CPR and Cerebral Perfusion

- Post-resuscitation cerebral perfusion is significantly compromised as a result of vasoconstriction and no-reflow phenomenon, thus leading to prolonged ischemic injury and neurological dysfunction.
Hypothesis

- Remote limb ischemic post-conditioning (RLIP) improves survival and neurological outcomes via
  - *In-situ* activation of Akt-eNOS-NO in the limb artery
  - Increased NO generation from PC limb vessels with release to the systemic circulation
  - Augmentation of cerebral blood flow and tissue perfusion by NO-mediated vasodilatation
  - Potential *remote* up-regulation of Akt-eNOS in the brain
Methods

- Well-established rat asphyxia cardiac arrest and CPR model
  - After surgical preparation, the rat was subjected to 6 min of asphyxia followed by ~1 min of CPR, with 1 dose of epinephrine (0.1 mg/Kg).
Methods

Study Designs

Asphyxia, ROSC

Standard CPR

CPR

RLIP

(5 min limb ischemia + 5 min reperfusion) X 3 cycles

RLIP + L-NAME

(5 min limb ischemia + 5 min reperfusion) X 3 cycles
L-NAME (10 mg/Kg) pretreated 1 h before cardiac arrest
Methods

- Hemodynamics continuously monitored
- Cerebral tissue perfusion continuously measured by OxyFlo sensors
- Blood sampled at 1 h for measurement of
  - Plasma nitrate/nitrite (Cayman colorimetric assay kit)
- All catheters and ET tube were removed at 4 h, with left hind limb artery, aorta and brain harvested for detecting
  - Phosphorylation of endothelium-derived nitric oxide synthase (eNOS)
  - Phosphorylation of protein kinase B (Akt)
- In a subgroup of rats with minimal invasive procedures
  - Survival continuously followed up to 3 days
  - Neurological scores measured at 4, 24, 48 and 72 hours
Measurement of Cerebral Blood Flow

- Cerebral blood flow was continuously measured and monitored by OxyFlo
Neurological Outcomes

- Neurological scores were evaluated at 4, 24, 48 and 72 hours post-resuscitation, and compared among groups.

Table 1: Neurological scoring of rat after resuscitation

<table>
<thead>
<tr>
<th>Level of consciousness</th>
<th>Corneal reflex</th>
<th>Respirations</th>
<th>Righting reflex</th>
<th>Coordination</th>
<th>Movement/activity</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No reaction to pinching of tail</td>
<td>1. No blinking</td>
<td>1. Irregular breathing pattern</td>
<td>1. No turning attempts</td>
<td>1. No movement</td>
<td>1. No spontaneous movement</td>
<td>0</td>
</tr>
</tbody>
</table>
Study Outcome Measurements

Overall outcomes
- Survival up to 3 days
- Neurological scores

Cerebrovascular function
- Cerebral blood flow and tissue perfusion

Mechanistic exploration
- Nitric oxide (NO) in systemic circulation as indicated by plasma nitrate/nitrite levels
- Activation of Akt-eNOS in situ in the post-conditioning limb artery
- Activation of Akt-eNOS in remote target vital organs such as brain
Methods

• **Statistical Analysis**
  – Serial changes analyzed by two-way ANOVA repeated measures method
  – Survival expressed by Kaplan-Meier survival curve and analyzed by Log-rank test
Results
Blood Pressure during and after CPR

![Graph showing blood pressure changes during CPR.](image)

- **mmHg**
- **Time (min)**

- **SCPR**
- **RLIP**
Cerebral Blood Flow and Tissue Perfusion

Folds of Baseline

- SCPR
- RLIP

Time (min)
Neurological Outcomes

* P < 0.05
*** P < 0.001
Plasma Nitrate/Nitrite

![Graph showing plasma nitrate/nitrite levels with baseline, SCPR, and RLIP groups.](image)
Phosphorylated-eNOS of PC Hind Limb Artery

p-eNOS  eNOS  β-actin

Fold of Baseline

Baseline  SCPR  RLIP

*
Phosphorylated-Akt of PC Hind Limb Artery

- p-Akt
- Akt
- β-actin

Fold of Baseline

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>SCPR</th>
<th>RLIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td>1.0</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Akt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates statistical significance.
Phosphorylated eNOS and Akt of Aorta

Phosphorylated eNOS and Akt of Aorta

Fold of Baseline

Fold of Baseline

p-eNOS

p-Akt

eNOS

Akt

β-actin

β-actin

p-eNOS

p-Akt

eNOS

Akt

β-actin

β-actin

p-eNOS

p-Akt

eNOS

Akt

β-actin

β-actin

p-eNOS

p-Akt

eNOS

Akt

β-actin

β-actin

p-eNOS

p-Akt

eNOS

Akt

β-actin

β-actin
Phosphorylated eNOS of Brain

- $\rho$-eNOS
- eNOS
- $\beta$-actin

**

**

Folds of Baseline

Baseline SCPR RLIP

Baseline | SCPR | RLIP
---|---|---
1.0 | 1.2 | 1.5

**

Phosphorylated eNOS of Brain
Phosphorylated Akt of Brain

p-Akt
Akt
β-actin

Folds of Baseline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>SCPR</th>
<th>RLIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td>1.0</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Akt</td>
<td></td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td>1.6</td>
</tr>
</tbody>
</table>

* indicates a significant difference.
Role of Nitric Oxide?

- Co-treatment of NO synthase inhibitor L-NAME (10 mg/kg) with PC, with measurement of
  - Plasma NO as indicated by nitrate/nitrite
  - Cerebral blood flow and tissue perfusion
  - Limb arterial phosphorylated eNOS/Akt
  - Brain phosphorylated eNOS/Akt
  - Survival outcome
  - Neurological scores
Plasma Nitrate/Nitrite

The graph shows the plasma nitrate/nitrite levels in different conditions: Baseline, SCPR, RLIP, and RLIP+LN. The y-axis represents the micromolar (μM) concentration, and the x-axis represents the different conditions. The graph indicates a significant increase in nitrate/nitrite levels in the RLIP condition compared to the other conditions, as indicated by the ** symbol. The RLIP+LN condition also shows a notable increase compared to Baseline and SCPR.
Phosphorylated-eNOS of PC Hind Limb Artery

Fold of Baseline

- **p-eNOS**
- **eNOS**
- **β-actin**

**Legend:**
- **Baseline**
- **SCPR**
- **RLIP**
- **RLIP+LN**

* indicates significant difference compared to Baseline.
Phosphorylated-eNOS of Brain

p-eNOS
eNOS
β-actin

Folds of Baseline

**

Baseline SCPR RLIP RLIP+LN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

p-eNOS

Baseline SCPR RLIP RLIP+LN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Folds of Baseline

**

p-eNOS

Baseline SCPR RLIP RLIP+LN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Folds of Baseline

**

p-eNOS

Baseline SCPR RLIP RLIP+LN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Phosphorylated-eNOS of Brain
Cerebral Tissue Perfusion

Folds of Baseline

- SCPR
- RLIP
- RLIP+LN

Time (min)
Blood Pressure

mmHg

Time (min)
Survival

Survival Rate vs Time (hours)

- SCPR
- RLIP
- RLIP+LN

Time (hours)
Neurological Outcomes

** P < 0.01
*** P < 0.001

6hr

24hr

48hr

72hr

SCPR  RLIP  RLIP + LN

SCPR  RLIP  RLIP + LN

SCPR  RLIP  RLIP + LN

SCPR  RLIP  RLIP + LN
Discussion

• Activation of *in situ* Akt-eNOS-NO pathway in the PC limb vessels results in *in situ* increase of NO.

• Release of NO into circulation leads to vasodilatation not only in PC limb but in *remote* organs/tissues such as brain.

• Such NO-mediated vasodilatation ameliorates the usually compromised cerebral perfusion in the post-resuscitation phase.

• Similar vasodilatation effect could be seen in other vital organs such as heart that improves myocardial perfusion and mitigates post-resuscitation myocardial dysfunction.
Discussion

• Akt-eNOS pathway has been shown to play important roles mediating intracellular anti-apoptotic signaling.

• Activation of Akt-eNOS signaling in the brain is consistent with previous studies showing that RLIP reduces apoptotic neuronal death after global I/R injury.

• The mediators and routes of remote Akt-eNOS activation need further studies.

Mechanisms of RLIP Neuro-protection

Remote Limb Ischemic Post-conditioning (RLIP)

- **In situ** activation of Akt-eNOS
  - Increase of NO generation
  - Release of NO to systemic circulation

- **Remote** activation of Akt-eNOS in vital organs (e.g. brain)
  - Anti-apoptosis Signaling
  - Decreased Apoptosis

- **Remote** Vasodilatation of **Remote** vital organs such as brain
  - Enhanced tissue perfusion
  - Decreased ischemic injury

- Decreased neuronal death and improved prognosis
Clinical Implications

• In contrast to pre-conditioning or whole-body post-conditioning, RLIP is theoretically safer and technically more feasible in the clinical settings.

• Clinical trials with RLIP by applying pressure cuff(s) in the limb(s) for cyclic interruption of limb circulation is feasible.
Conclusions

- RLIP after cardiac arrest and CPR protects the brain via enhancement of the compromised cerebral blood flow and tissue perfusion, thereby improving the survival and neurological outcomes of the patients.

- The protection is in part mediated by
  - *In situ* release of NO via activation of the endothelial Akt-eNOS-NO pathway in the PC limb vessel.
  - *Remote* activation of Akt-eNOS signaling in the brain, which may in turn trigger downstream anti-apoptotic pathway.
Thank You for Your Attention