AWARD NUMBER: W81XWH-16-1-0606

TITLE: Application of Combined Cardioprotective Agents to Preserve Organ Function and Improve Survival during Experimental Hemorrhagic Shock

PRINCIPAL INVESTIGATOR: Robert A. Kloner MD, PhD

CONTRACTING ORGANIZATION: Huntington Medical Research Institutes
Pasadena, CA 91101

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Fort Detrick, Maryland 21702-5012

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Application of Combined Cardioprotective Agents to Preserve Organ Function and Improve Survival during Experimental Hemorrhagic Shock

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6. AUTHOR(S)

Dr. Robert A. Kloner

E-Mail: robert.kloner@hmri.org

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5e. TASK NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Huntington Medical Research Institues

686 South Fair Oaks Avenue
Pasadena, CA 91105

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
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12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We investigated the effects of hypothermia treatment alone and in combination with experimental bilateral lower limb remote ischemic preconditioning (RIPC) in rats undergoing experimental hemorrhagic shock. Previously we showed that RIPC alone improved survival. In the setting of hypothermia alone, 4 of 15 (26.7 %) rats in the control group and 11 of 16 (68.8 %; p = 0.032) rats in the hypothermia group survived at 6 weeks. pO2 remained essential normal and levels of potassium, chloride, and lactate were lower in hypothermia group. In the combination study, 1 of 10 (10 %) rats in the control group and 7 of 11 (63.6 %; p = 0.024) rats in the hypothermia plus RIPC group survived at 6 weeks. The neutrophil-to-lymphocyte ratio (NLR) was significantly lower in Hypothermia plus RIPC. NLR has been reported to be strongly associated with early mortality in patients with severe hemorrhage and represents disease severity. We concluded that hypothermia alone and combination with RIPC significantly improved long term survival in rats subjected to hemorrhagic shock. Hypothermia and RIPC did not show survival rates greater than hypothermia alone.

15. SUBJECT TERMS

Hemorrhagic shock, therapeutic hypothermia, therapeutic hypothermia plus remote ischemic preconditioning, fixed pressure hemorrhage

16. SECURITY CLASSIFICATION OF: u			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	24	

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1. INTRODUCTION:

Hemorrhagic shock remains a major cause of mortality and morbidity on the battlefield. Even with restoration of blood volume, organs subjected to hemorrhagic shock can develop ischemia/reperfusion injury and fail. This study aims to develop new therapeutic approaches to improve survival and protect vital organs during and after hemorrhagic shock. The therapies with proven cardio-protective properties include: (1) remote ischemic preconditioning, which could be given prophylactically (simple and cost-effective repetitive inflations and deflations of a blood pressure cuff on the arm) to soldiers prior to going into high risk combat situations; (2) the mitochondrial protective agent SS31, which could also be administered prophylactically; and (3) therapeutic hypothermia, which could be produced with a ThermoSuit device (already FDA approved for hyperthermia) to rapidly cool the body and protect vital organs in case the injury occur. These therapies, alone or in combination, will be investigated to improve overall survival and protect vital organs from ischemia/reperfusion injury of hemorrhagic shock (vs. placebo) in a standardized experimental model of fixed pressure hemorrhage in male and female adult Sprague Dawley rats.

2. KEYWORDS:

Hemorrhagic shock; remote ischemic preconditioning; mitochondrial protective agent; therapeutic hypothermia; fixed pressure hemorrhage.

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To test the hypothesis that prophylactic remote ischemic preconditioning improves long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after experimental hemorrhagic shock, created by withdrawing blood and later reinfusing it, in Sprague Dawley rats of both sexes. **The major tasks** are: (1) from 1 to 6 months, instrument rats and perform studies for Specific Aim 1 (n = ~90 rats); (2) from 7 to 8 months, collect and collate data; (3) in 9th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether remote ischemic preconditioning improves long term survival and improves the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. This study is 100% completed.

Specific Aim 2: To test the hypothesis that prophylactic administration of mitochondrial protective agent, SS31, improves long term survival and the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. **The major tasks** are: (1) from 10 to 15 months, instrument rats and perform studies for Specific Aim 2 (n = ~90 rats); (2) from 16 to 17 months, collect and collate data; (3) in 18h month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether prophylactic administration of mitochondrial protective agent, SS31, improves long term survival and improves the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. This study is 100% completed.

Specific Aim 3: To test the hypothesis that moderate hypothermia administered in the setting of hemorrhagic shock will improve long term survival and preserve the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. **The major tasks** are: (1) from 19 to 24 months, instrument rats and perform studies for Specific Aim 3 (n = ~90 rats); (2) from 25 to 26 months, collect and collate data; (3) in 27th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether moderate hypothermia administered in the setting of hemorrhagic shock will improve long term survival and preserve the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. We have completed about 75% of this study. Biochemistry and histology are pending.

Specific Aim 4: To test the hypothesis that a combination of promising therapies tested in aims 1, 2, and/or 3, which have been shown to have a positive effect, will have additive (or synergistic) effects on survival and organ preservation in the setting of hemorrhagic shock. **The major tasks** are: (1) from 28 to 33 months, instrument rats and perform studies for Specific Aim 4 (n = ~90 rats); (2) from 34 to 35 months, collect and collate data; (3) in 36th month, analyze data to assess survival, organ function and structure and mitochondrial function. **Milestone(s) Achieved** – Determine whether a combination of promising therapies tested in aims 1, 2, and 3 have additive (or synergistic) effects on survival and organ preservation in the setting of hemorrhagic shock. This study is being completed and will be finished during year 3 and 4. Hemodynamic and Echo analysis are pending. Biochemistry and histology are pending.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

This annual report covers 1 Sep 2018 – 30 Aug 2019, and focuses on the major goals listed in the **Specific Aim 3 and 4**.

In the study of **Specific Aim 3,** the hypothesis that moderate hypothermia administered in the setting of hemorrhagic shock will improve long term survival and preserve the function and structure of the vital organs (heart, brain, lung, kidney and liver) after an episode of hemorrhagic shock in an experimental rat model. Sprague-Dawley rats (both genders) were randomly assigned to hypothermia (n = 16) or control group (n = 15). Fixed-pressure hemorrhagic shock was induced in the rats. The rats were anesthetized with intraperitoneal ketamine and xylazine (90mg/kg and 10mg/kg), and were heparinized with 500 U/kg heparin. Hemorrhagic shock was induced by removing blood via the carotid artery catheter to attain a mean blood pressure of 30 mm Hg. Mean blood pressure (MBP) was maintained at 30 mm Hg for 30 minutes and then the collected blood was returned over the next 30 minutes. Hypothermia using the Thermosuit was started 5 minutes after MBP reached 30 mmHg. Core temperature was maintained at ~ 32 °C until blood volume was fully restored, after which the rats were allowed to warm back to normal temperature. In the control group, body temperature was maintained at ~ 37°C. Arterial blood samples were collected 1 hour after resuscitation with shed blood. At 60 minutes after returning the shed blood and closely monitoring the animals, the catheters were removed from the blood vessels and the rats were allowed to recover from anesthesia; and then allowed to survive for 6 weeks. At 1 hour after resuscitation of hemorrhagic shock, pO2 (partial pressure of oxygen) remained essentially normal in hypothermia (117.8 ± 9.9) vs control group (81.6 ± 4.8; p=0.004). The rats in the control group had significantly elevated potassium, chloride and lactate levels and more negative base excess compared to rats in the hypothermia group. At 6 weeks, 4 of 15 (26.7 %) rats in the control group and 11 of 16 (68.8 %; p = 0.032) rats in the hypothermia group survived. In conclusion, hypothermia significantly improved long term survival in rats subjected to hemorrhagic shock. Hypothermia influenced the levels of blood gases and blood counts, favoring hypothermia over control, which may help explain the protective role of hypothermia in hemorrhagic shock.

in **Specific Aim 4.** the hypothesis that a combination of promising therapies tested in aims 1, 2, and/or 3, which have been shown to have a positive effect, will have additive (or synergistic) effects on survival and organ preservation in the setting of hemorrhagic shock. Sprague-Dawley rats (both genders) were randomly assigned to hypothermia plus preconditioning group (n = 11) or control group (n = 10). Fixed-pressure hemorrhagic shock was induced in the rats. The rats were anesthetized with intraperitoneal ketamine and xylazine (90mg/kg and 10mg/kg), and were heparinized with 500 U/kg heparin. Hemorrhagic shock was induced by removing blood via the carotid artery catheter to attain a mean blood pressure of 30 mm Hg. Mean blood pressure (MBP) was maintained at 30 mm Hg for 30 minutes and then the collected blood was returned over the next 30 minutes. Preconditioning was induced by 4 cycles of inflating small cuffs around the femoral arteries to 200 mmHg for 5 minutes, followed by 5-minute deflation of the cuffs prior to hemorrhagic shock. Hypothermia was started 5 minutes after MBP reached 30 mmHg. Core temperature was maintained at ~ 32 °C until blood volume was fully restored, after which the rats were allowed to warm back to normal temperature. In the control group, body temperature was maintained at ~ 37°C. Arterial blood samples were collected 1 hour after resuscitation with shed blood.

The rats were allowed to recover from anesthesia; and then allowed to survive for 6 weeks. At 1 hour after resuscitation of hemorrhagic shock, the neutrophil-to-lymphocyte ratio (NLR) was significantly lower in Hypothermia plus RIPC (0.19 ± 0.02) versus the control group (0.33 ± 0.02; p=0.001). The NLR is an easily accessible biomarker, which is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. NLR has been reported to be strongly associated with poor prognosis in severe hemorrhage. At 6 weeks, 1 of 10 (10 %) rats in the control group and 7 of 11 (63.6 %; p = 0.024) rats in the hypothermia plus RIPC group survived at 6 weeks. In conclusion, hypothermia combination with RIPC significantly improved long term survival in rats subjected to hemorrhagic shock. Hypothermia and RIPC did not show survival rates greater than hypothermia alone. Hemodynamic and Echo data analysis are pending. Various biochemical studies and histologic analyses are pending.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

During the year of 1 Sep 2018 – 30 Aug 2019, the present studies have resulted in 1 manuscript and 3 abstracts that were presented for American Heart Association Scientific Sessions 2018 (Resuscitation Science Symposium), which was held in Chicago, IL, November 10-11, 2018.
Manuscript:

1. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Improved Long-term Survival with Remote Limb Ischemic Preconditioning in a Rat Fixed-Pressure Hemorrhagic Shock Model. Cardiovasc Drugs Ther. 2019 Apr; 33(2):139-147.

Abstracts:

1. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Remote limb ischemic preconditioning improves short and long term survival and maintains intravascular blood volume during resuscitation of hemorrhagic shock. Circulation. 2018; 138: A186.
2. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Effects of anesthetic agents on the hemodynamic stabilization and long term survival in an experimental model of hemorrhagic shock. Circulation. 2018; 138: A187.
3. Shi J, Dai W, Carreno J, Hale SL, Kloner RA. Effects of anesthetic agents on blood parameters in rats with hemorrhagic shock. Circulation. 2018; 138: A191.

And another 3 abstracts have been submitted to the American Heart Association Scientific Sessions 2019 (ReSS), which will be held in Philadelphia, PA, November 16-17, 2019

1. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner. Therapeutic hypothermia alone and in combination with preconditioning improves long term survival during resuscitation of hemorrhagic shock.
2. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Effects of Therapeutic Hypothermia on Blood Parameters in Rats with Acute Hemorrhagic Shock.
3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Therapeutic Hypothermia Alone and Combination with Preconditioning Blunt Inflammation in Experimental Hemorrhagic Shock.

In addition, we are in the process of writing up several full length manuscripts and hope to have them submitted to a peer review journals in 2019-2020.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During next reporting period, we will complete the data analysis in Specific Aim 2 to 4, which include hemodynamic measurements and Echo parameter. We will also complete all the analysis on histology and histochemistry: inflammation markers (TNF alpha, IL-6, IL-1, MCP), myeloperoxidase activity, GSH/GSSG ratio and tissue analysis for mitochondrial gene expression and function. EM will be performed for mitochondrial morphology.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Therapeutic hypothermia administered in the setting of hemorrhagic shock improved long term survival. Therapeutic hypothermia influenced the levels of blood gases and blood counts, favoring hypothermia over control group. This study should have a major clinical implication for the military: should the injury occur, then therapeutic hypothermia can be added for rapid additional protection to improve survival.

Therapeutic hypothermia combined with remote ischemic preconditioning significantly improves long term survival and decreases inflammation in rats subjected to hemorrhagic shock. Hypothermia combined with remote ischemic preconditioning did not show survival rates greater than hypothermia alone. It suggested that the combinations of these two therapies do not have additive (or synergistic) effects on survival in the setting of hemorrhagic shock.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

It could be that therapeutic hypothermia and combination with preconditioning work in this model that it might work in other types of shock that include not only hypotension but tissue trauma, septic shock, and other forms of ischemia/reperfusion injury to organs.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

We observed an improved survival with therapeutic hypothermia. The result is suggestive of a benefit, and could translate to a clinical study whereby patients in hemorrhagic shock receive therapeutic hypothermia.

This finding could be applied to survival following not only hemorrhage in the battle field, but could be applied to civilians following car accidents or gunshot wounds.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

During the year of 1 Sep 2018 – 30 Aug 2019, we did not make any changes.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

None

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

There were no significant changes in use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents

No change in biohazards.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

One manuscript has been published in the journal of Cardiovascular Drugs Therapy.

1. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Improved Long-term Survival with Remote Limb Ischemic Preconditioning in a Rat Fixed-Pressure Hemorrhagic Shock Model. Cardiovasc Drugs Ther. 2019 Apr; 33(2):139-147. Acknowledgement of federal support: yes.

There are 3 abstracts that have been published in the journal of Circulation.

1. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Remote limb ischemic preconditioning improves short and long term survival and maintains intravascular blood volume during resuscitation of hemorrhagic shock. Circulation. 2018; 138: A186.
2. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Effects of anesthetic agents on the hemodynamic stabilization and long term survival in an experimental model of hemorrhagic shock. Circulation. 2018; 138: A187.
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There are another 3 abstracts that has been submitted in the journal of Circulation.

1. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner. Therapeutic hypothermia alone and in combination with preconditioning improves long term survival during resuscitation of hemorrhagic shock.
2. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Effects of Therapeutic Hypothermia on Blood Parameters in Rats with Acute Hemorrhagic Shock.
3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Therapeutic Hypothermia Alone and Combination with Preconditioning Blunt Inflammation in Experimental Hemorrhagic Shock.

Note: All accepted presentations (posters) at scientific meeting acknowledged federal support.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None yet

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

During the year of 1 Sep 2018 – 30 Aug 2019, the present studies have resulted in 3 abstracts that are presented in 2018 American Heart Association Scientific Sessions 2018 (ReSS), which was held in Chicago, IL, November 10-11, 2018

1. Dai W, Shi J, Carreno J, Hale SL, Kloner RA. Remote limb ischemic preconditioning improves short and long term survival and maintains intravascular blood volume during resuscitation of hemorrhagic shock. Circulation. 2018; 138: A186.
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And another 3 abstracts are submitted in the American Heart Association Scientific Sessions 2019 (ReSS), which will be held in Philadelphia, PA, November 16-17.

1. Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A. Kloner. Therapeutic hypothermia alone and in combination with preconditioning improves long term survival during resuscitation of hemorrhagic shock.
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3. Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner. Therapeutic Hypothermia Alone and Combination with Preconditioning Blunt Inflammation in Experimental Hemorrhagic Shock.

(The 1 manuscript and 6 abstracts are attached in Section 9 - APPENDICES)

Note: Several manuscripts based upon the data presented at national meeting and planned for writing and submitting in 2019-2020.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  None.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  Not applicable

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Not applicable

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

OTHER: Reports at scientific sessions. 3 abstracts were presented in 2018 American Heart Association Scientific Sessions 2018 (ReSS), which was held in Chicago, IL, November 10-11, 2018; another 3 abstracts are submitted in the American Heart Association Scientific Sessions 2019 (ReSS), which will be held in Philadelphia, PA, November 16-17.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award).*

1. Name: Robert A. Kloner
Project Role: Principle investigator
Nearest person month worked: 6 months (50% per year)
Contribution to Project: Dr. Kloner has performed work in the area of study design, data collection and analysis, report and manuscript writing.

2. Name: Wangde Dai
Project Role: Senior investigator
Nearest person month worked: 9.6 months (80% per year)
Contribution to Project: Dr. Dai has performed work in the animal studies, data collection and analysis, report and manuscript writing.

3. Name: Jianru Shi
Project Role: Senior investigator
Nearest person month worked: 5.4 months (45% per year)
Contribution to Project: Dr. Shi has performed work in the area of blood and tissue sampling, data collection and analysis, report and manuscript writing.

4. Name: Sharon Hale
Project Role: Research Associate
Nearest person month worked: 1 month (8.3% per year)
Contribution to Project: Mrs Hale has performed work in the area of study design, data collection and analysis, report and manuscript writing.

5. Name: Juan Carreno
Project Role: Veterinary Support, Head of Vivarium, Research Assoc.
Nearest person month worked: 0.6 month (5% per year)
Contribution to Project: Dr. Carreno has performed work in the area of animal handling and caring, data collection and analysis, report and manuscript writing.

6. Name: Jesus Chavez
Project Role: Histology technician
Nearest person month worked: 4.2 month (35 % per year)
Contribution to Project: Mr. Chavez has performed work in the area of tissue collection, processing, sectioning, and various staining.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Robert A. Kloner:
Tobacco-Related Disease Research Program (TRDRP; University of California). PI: Robert A. Kloner. Effects of Cigarette Smoking and Vaping on Heart Attack. Proposal Number (pC ID): 587712; Award number; 281R-0057 ($1,066,980). 2018-2021. 20% effort.

NHLBI. R01 HL144258. PI: Robert A. Kloner. The effect of electronic cigarettes on young versus old normal hearts and pathologic hearts ($1,839,340). 2018-2022. 10% effort.

Wangde Dai:
Tobacco-Related Disease Research Program (TRDRP; University of California). PI: Robert A. Kloner. Effects of Cigarette Smoking and Vaping on Heart Attack. Proposal Number (pC ID): 587712; Award number; 281R-0057 ($1,066,980). 2018-2021. 10% effort.

NHLBI. R01 HL144258. PI: Robert A. Kloner. The effect of electronic cigarettes on young versus old normal hearts and pathologic hearts ($1,839,340). 2018-2022. 10% effort.

Jianru Shi:
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What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:

Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

None.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
QUAD CHARTS:

9. APPENDICES:

Below please find the 1 manuscript, 3 accepted abstracts and 3 submitted abstracts

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ORIGINAL ARTICLE

95, USA

In the clinical setting, lifesaving approaches to treat hemorrhagic shock include early recognition of shock, rapid control of the source of hemorrhage, and restoration of volume and oxygen-carrying capacity, in the attempt to limit the depth and duration of the shock state before it becomes irreversible [5]. Resuscitation treatment focuses primarily on fluid replacement using oxygen-carrying blood substitutes, hypertonic solutions, colloid solutions, and crystalloids [6].

Many experimental studies have tested the administration of various fluids as treatment for hypovolemic shock in a variety of models (see [7, 8] for reviews). However, a different approach would be to increase tissue tolerance to hemorrhagic shock. Until recently, this strategy has remained largely unexplored.

One such approach might be to enhance ischemic tolerance using remote ischemic preconditioning (RIPC). Over the past 2 decades, numerous experimental and clinical studies have demonstrated that RIPC is a therapeutic, noninvasive intervention capable of conferring multi-organ protection against acute ischemia/reperfusion injury [9]. RIPC, which was originally characterized as an interaction between two separate coronary vascular territories [10], is the phenomenon whereby the controlled induction of nonlethal and transient ischemia in one organ or tissue increases ischemic tolerance in a distant vital organ or tissue against prolonged ischemia-reperfusion-induced injury [11]. Although the underlying molecular mechanism of the protective effects remains unknown, remote limb ischemic preconditioning is well known to provide cardioprotection [10, 12], neuroprotection [13], pulmonoprotection [14], hepatoprotection [15], and gastrointestinal protection [16, 17]. Therefore, preconditioning might be a potential therapeutic option to provide benefit against hemorrhagic shock-induced multi-organ injury.

Some experimental studies have been performed to test this hypothesis [18–23], and data showed a benefit from preconditioning in models of hemorrhagic shock. However, short-term survival was assessed in only a few of these studies and long-term survival was not assessed at all. Therefore, the purpose of the present study was to investigate whether RIPC could improve long-term survival in a rat model of hemorrhagic shock/resuscitation.

Methods

The present study was approved by the Institutional Animal Care and Use Committees at Huntington Medical Research Institutes and was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington DC, revised 2011). All animals were housed in environmentally controlled rooms with temperature at 22 ± 2 °C and 12-h light-dark cycles, and allowed free access to standard laboratory chow and water. Prior to surgery, the rats were acclimatized for a minimum of 5 days after arrival at the laboratory. We specifically chose the rat model of hemorrhagic shock as there is already a body of literature based on this model [24], and in pilot studies, we could show that a duration of 30 min of blood pressure lowered to 30 mmHg resulted in significant mortality of about 20–30%, which allows for room for therapeutic improvement. In addition, many of the kinds of studies that previously required larger animals can be done in the rodent model including hemodynamic measurements and echocardiography.

Surgical Procedures for Hemorrhagic Shock Model

Age-matched Sprague-Dawley rats (both genders, mean weight 217 ± 3 g for females and 337 ± 5 g for males), obtained from Charles River, Inc., were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg). A cannula was inserted into the trachea and the cannula attached to a respirator; the rats were ventilated with room air at 60 strokes/min and 10 ml/kg tidal volume. Body temperature was maintained at 37 °C with a heating pad. Under aseptic conditions, the left femoral artery, carotid artery, and jugular vein were dissected using a minimal dissection technique, distally ligated, and cannulated with a polyethylene catheter (PE-50) filled with heparinized saline (8 IU/ml) to avoid local coagulation. The femoral artery catheter was connected to a pressure transducer to continuously monitor the arterial blood pressure and heart rate, and the carotid artery catheter was used for shed blood withdrawal to induce pressure-controlled hemorrhagic shock. The jugular vein catheter was used to reinfuse the shed blood. Lead II of the electrocardiogram (ECG) was continuously recorded. After the rats were heparinized with intravenous heparin (500 U/kg), the shed blood was withdrawn into a heparinized syringe over a period of 10 min using a syringe pump and kept at room temperature. The mean blood pressure was reduced to a target value of ~ 30 mmHg and maintained at that pressure for 30 min by withdrawing or infusing small amounts of blood. Shed blood volume was expressed as a percentage of calculated total blood volume [estimated from 6.12 ml/100 g body weight] [18]. After 30 min of shock, the rats were resuscitated with the reinfusion of total shed blood over a 30-min period, and the rats were continuously monitored for another 30 min. At 1 h after initiation of resuscitation, the catheters were removed from the femoral artery, carotid artery, and jugular vein and the blood vessels tied off. The neck and groin incisions were closed in layers. The rats were returned to their cages to allow recovery from anesthesia. Postoperative analgesia (buprenorphine, 0.01 mg/kg body weight, subcutaneous) was maintained for 2 days. They were observed 6 weeks for survival.

Experimental Design

Fifty-three rats were randomized into RIPC and control groups. Randomization was achieved by placing folded pieces of paper with group assignment in a jar and drawing blindly after surgical preparation was complete. Rats in the RIPC group underwent bilateral hind-limb ischemia/reperfusion for 4 consecutive cycles of 5 min of ischemia followed by 5 min of reperfusion, prior to blood withdrawal to induce hemorrhagic shock. After the last 5 min of reperfusion, shock (30 mmHg) was achieved in about 10 min of bleeding in both groups. Bilateral conditioning was used because it has been shown to be effective in various models [11, 25–27]. RIPC was delivered by placing the blood pressure cuffs (one cuff on each hind leg) at the inguinal level, and ischemia was induced by inflating the cuff with air to 200 mmHg. Reperfusion was initiated by deflating the cuffs. In the control group, all procedures were followed as in RIPC group except the cuff pressures were inflated only to 30 mmHg.

Cardiac Function

Transthoracic echocardiographic imaging was performed using a 15-MHz linear array transducer of a Philips ultrasound system at baseline prior to bleeding, 5 min before resuscitation with shed blood, and 1 h after initiation of resuscitation. Echocardiograms were analyzed and measurements calculated post hoc in a blinded fashion. Two-dimensional parasternal short-axis views and two-dimensional targeted M-mode tracings were taken to determine diastolic and systolic internal dimensions of the left ventricle (LVIDd and LVIDs, mm) and left ventricular fractional shortening (LVFS, %), respectively.

Blood Gas Analyses

Blood samples of 0.3 ml were collected from the carotid arterial catheter at 1 h after initiation of resuscitation. Blood pH, arterial partial pressure of carbon dioxide ($PaCO_2$), arterial partial pressure of oxygen (PaO_2), Na, K, Cl, iCa, glucose, and lactate concentration (Lac) were measured with a blood gas analyzer. In addition, blood was collected for cell counts, platelet count, and other electrolytes and chemistries.

Analyses of C-reactive Protein and Cytokines

At 48 h and 6 weeks resuscitation, serum c-reactive protein (CRP) levels were determined using the Rat CRP ELISA Kit (RayBiotech, Norcross, GA, USA). Serum TNFα, IL-1α, IL-1β, and IL-6 were measured using the multiplex cytokines kit (IDEXX laboratories, Columbia, MO, USA) according to the manufacturer's instructions.

Tissue Histological Analysis

At 6 weeks after hemorrhagic shock, all surviving rats were deeply anesthetized. To detect areas of microvascular damage in the tissues, thioflavin S was injected into the jugular vein. The rats were euthanized by an intravenous injection of KCl. Following euthanasia, heart, brain, lungs, liver, and kidney were isolated, weighed, and photographed under white light, and under ultraviolet light to detect thioflavin S perfusion defects that demarcate microvascular obstruction. Triphenyltetrazolium chloride staining was used to identify necrosis (white areas) versus viable tissue (brick red areas). The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. The processed tissues were sectioned into 5-μm slices and were used for hematoxylin and eosin staining for tissue structure, and picrosirius red staining to assess fibrosis.

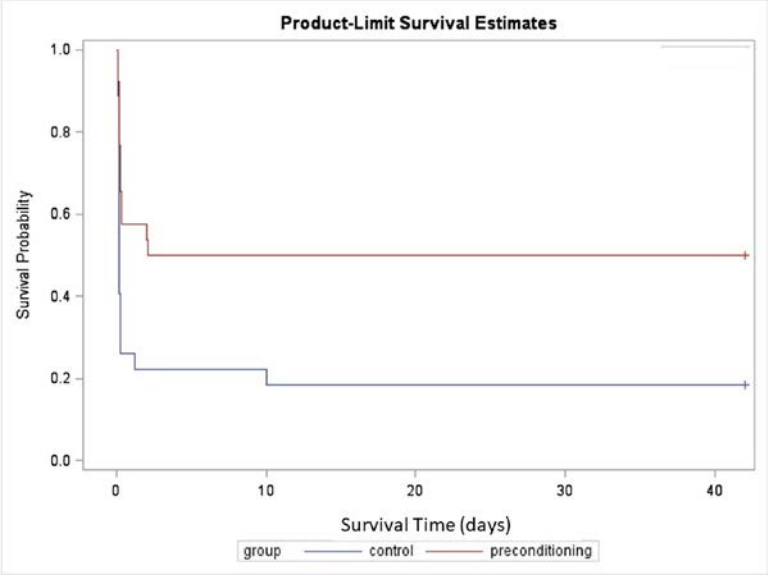
Statistical Analyses

Statistical analyses were performed with SAS v.9.4 software. Parametric data are reported as mean ± SEM. Non-parametric data (cytokines) were analyzed using rank sum testing and expressed as median values. *T* tests were used for blood gas and chemistry variables. A linear mixed-models analysis for repeated measures was used to analyze hemodynamic variables, testing for a group × time interaction. Differences between specific time points of hemodynamic variables were determined by post hoc contrasts from the same mixed models. Survival analysis was performed by log-rank testing. Values were considered significant at $p < 0.05$.

Results

Survival

A total of 14 female and 13 male rats in the control group and 12 female and 14 male rats in the RIPC group were used in this study. At 6 weeks, 5 of 27 (19%) rats in the control group and 13 of 26 (50%; $p = 0.021$) rats in the RIPC group survived. The survival curves for the two groups diverged within the first day (Fig. 1), indicating that RIPC improved early survival. This benefit on survival was maintained long term: RIPC significantly increased survival at 6 weeks. Survival data, analyzed by Log-rank test: $\chi^2(1) = 8.1$, $p = 0.004$, indicated that the two groups are significantly different from one another in their survival distributions. Most rats that failed to survive died within 24 h, but 1 rat died at 29 h and 1 at 10 days in the control group; 2 rats died at 2 days in the RIPC group. No seizures were observed in any of the animals. There was no significant difference in survival by gender at 6 weeks in either control or RIPC groups.



Shed Blood Volume

There was no difference in rat body weights between the two groups on the day of hemorrhagic shock (275 ± 13 g in control group and 281 ± 13 g in the RIPC group; $p = 0.72$). The total withdrawn blood volume (expressed as percentage of estimated total blood volume) was 42 ± 1% in the control group ($n = 27$), and 49 ± 19% in the RIPC group. There was no difference in the shed blood volume withdrawn to maintain the mean blood pressure ~ 30 mmHg during the shock phase ($p = 0.84$).

Hemodynamics During Shock and Resuscitation

There was a significant overall difference in mean blood pressures in the two groups (group × time interaction term, $p < 0.0001$) (Fig. 2). During the first 15 min after initiation of resuscitation, mean blood pressure increased in both groups and tended to be higher in the RIPC group. Then, the blood pressure gradually decreased in both groups. Overall, there was no significant difference in heart rate (group × time interaction term, $p = 0.6$). Pulse pressure was different between the two groups determined by the group × time interaction term ($p = 0.01$), but there was no specific time points showing a group difference (all p values greater than 0.4).

Cardiac Function

The diastolic and systolic internal dimensions of the left ventricle (LVIDd and LVIDs, mm) and percentage of left ventricular fractional shortening (LVFS, %) were similar in both groups at baseline. Blood withdrawal significantly decreased LVIDd and LVIDs and increased the LVFS in both groups. LVIDd, LVIDs, and the LVFS were comparable in the two groups 5 min before resuscitation with the shed blood. Shed blood reinfusion increased the LVIDd and LVIDs at 1 h after initiation of resuscitation in both groups, but LVIDd was significantly higher in the RIPC group suggesting that there was more circulating intravascular blood volume in the RIPC group than in the control group (Table 1). LVFS was not depressed during any of the measures in either group.

Arterial Blood Analyses

There were no statistically significant differences at 1 h after initiation of resuscitation in arterial blood gas values (Supplemental Data Table 1). PaO_2 and $PaCO_2$ levels were lower than those reported in normal rats, but there were no differences between controls and RIPC.

Blood counts and chemistries were similar in both groups measured at 1 h after initiation of resuscitation. Magnesium levels were slightly but significantly lower in the RIPC group; otherwise, there were no differences in other parameters between groups (see Supplemental Data Table 2).

Blood sampled at 48 h after resuscitation through a catheter in the tail vein (see Supplemental Data Table 3) demonstrated liver injury in both groups as indicated by elevated AST and ALT, but there were no significant differences between groups. Blood urea nitrogen (BUN) was within normal range

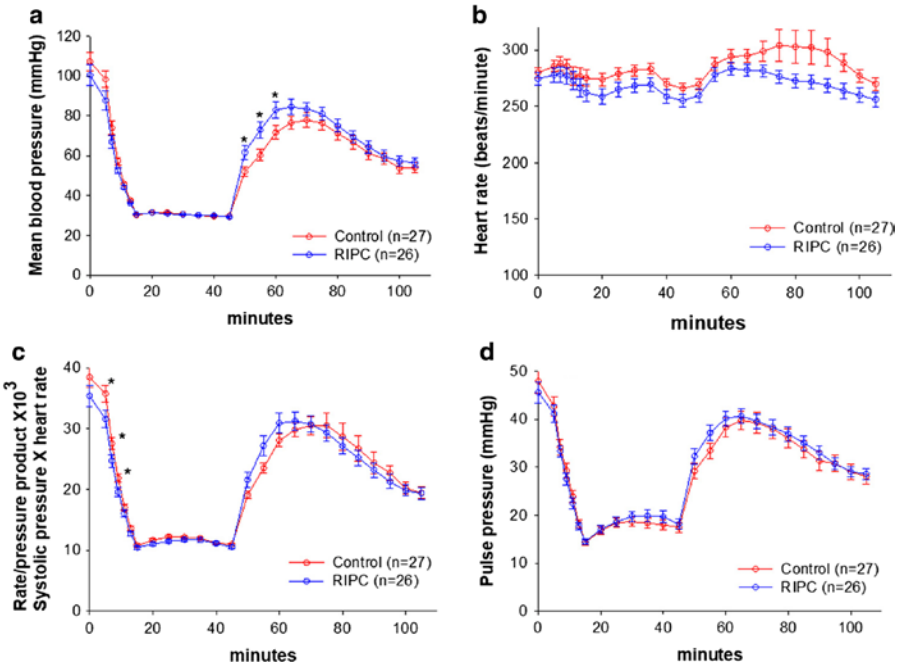


Fig. 2 Hemodynamic variables at baseline and during blood withdrawal, the shock phase, resuscitation, and observation phases. **a** The time course of mean arterial pressure is significantly different for the two groups (group × time interaction term, $p < 0.0001$; an asterisk indicates the time points, at which there is significant difference between the two groups). **b** The time course of heart rate is not significantly different for the two groups (group × time interaction term, $p = 0.6$). **c** The time course of rate/pressure product is significantly different for the two groups (group × time interaction term, $p = 0.0002$; an asterisk indicates the time points, at which there is significant difference between the two groups). **d** The time course of pulse pressure is significantly different for the two groups (group × time interaction term, $p = 0.01$), but there was no specific time points showing a group difference (all p values > 0.4 at different time points)

in the RIPC group (17.3 ± 1.2 mg/dl) but elevated in the control group (22.0 ± 1.7 mg/dl).

Table 1 Cardiac function (echocardiography)

	Control ($n = 27$)	RIPC ($n = 26$)	p value
Baseline			
Diastolic ID (mm)	6.7 ± 0.1	6.7 ± 0.1	0.83
Systolic ID (mm)	3.8 ± 0.1	3.8 ± 0.1	0.84
LVFS (%)	43.4 ± 1.1	44.2 ± 1.1	0.59
25 min of shock			
Diastolic ID (mm)	4.1 ± 0.1	3.9 ± 0.1	0.28
Systolic ID (mm)	2.2 ± 0.2	1.9 ± 0.2	0.19
LVFS (%)	47.8 ± 2.6	52.8 ± 2.8	0.20
1 h after blood resuscitation			
Diastolic ID (mm)	5.4 ± 0.1	5.8 ± 0.1	0.04
Systolic ID (mm)	2.7 ± 0.1	2.9 ± 0.1	0.46
LVFS (%)	49.6 ± 1.8	50.9 ± 1.9	0.64

ID, internal dimension; *LVFS*, left ventricular fractional shortening

C-reactive Protein and Cytokines

CRP levels were higher (non-statistically) in the RIPC group at 48 h (1118 ± 383 μg/ml) than at 6 weeks (448 ± 84). In the control groups, values were similar both at 48 h and 6 weeks (784 ± 416 and 761 ± 79 μg/ml, respectively). Median values for IL-1a, IL-1b, and IL-6 were similar in both groups at 48 h and 6 weeks. Serum TNF alpha levels were undetectable in both groups at 48 h and 6 weeks.

Histology of Tissues

At 6 weeks after hemorrhagic shock, gross pathology and light microscopy analyses were performed in the tissues from some surviving rats and showed brain infarction in 1 of 3 (33%) rats in the control group and 2 of 7 (29%) rats in the RIPC group. In brains without gross infarcts, the histology appeared normal in both groups (Fig. 3). Areas of microvascular damage (no-reflow) were not detected in the damaged brains or in other tissues. No evidence of myocardial, liver, or kidney damage were detected by either gross pathology or by microscopic

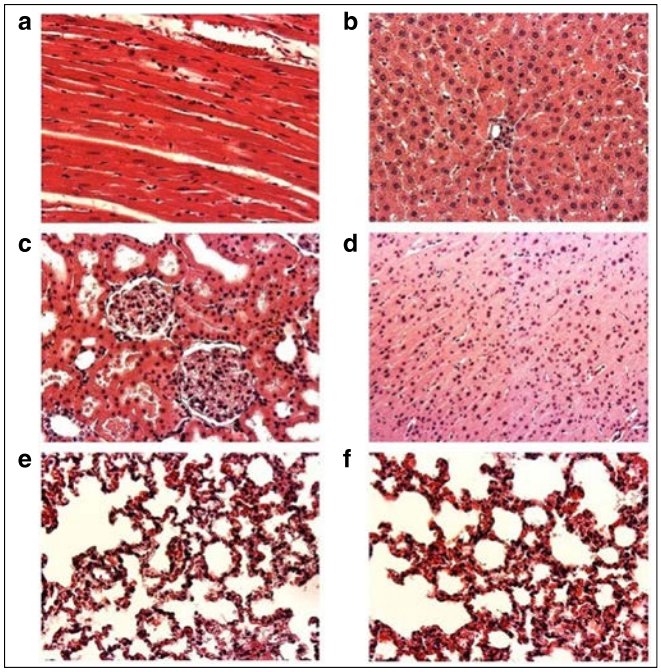


Fig. 3 Representative images of heart, liver, kidney, brain, and lung histology after H&E staining (original magnification × 20). **a** Section of heart in the control group. **b** Section of liver in the control group. **c** Section of kidney in the control group. **d** Section of brain in the control group. **e** Section of lung in the control group. **f** Section of lung in the RIPC group. The microscopic appearance of the heart, liver, and kidney was normal. In those brains without infarctions, the histology appeared normal. Lungs showed abnormalities in both groups as described in the "Histology of Tissues" section

analysis. Hematoxylin & eosin (H&E)-stained lung sections showed patchy lung lesions, including areas of thickened alveolar septa with mononuclear cell infiltration, disrupted alveolar morphology, and erythrocyte leaking into alveoli in control and RIPC groups. There was no difference in lung pathology between the control and RIPC groups. There were no architectural changes, inflammation, fibrosis, or necrosis in H&E-stained heart, kidney, or liver sections in either group.

Discussion

The major finding of the present study is that RIPC markedly improved short-term survival, after experimental hemorrhagic shock/resuscitation, and this benefit was maintained in the long term. Most deaths occurred within the first few days of shock, and animals that survived for the first few days survived long term. This suggests that if resuscitation is successful after acute bleeding, then there is usually no lethal consequences long term. While RIPC has been shown to be beneficial in numerous experimental and clinical studies including those of acute myocardial infarction and even stroke [28–31], there have been very few studies examining its effect in hemorrhagic shock and none that have assessed long-term survival.

This finding of a long-term benefit parallels data from a study performed in coronary artery bypass grafting patients who received RIPC prior to undergoing heart surgery [32]. In a follow-up study, all-cause mortality was significantly lower in the RIPC group at 5.83 years compared with the control group [33].

Numerous experimental and clinical studies have demonstrated that RIPC can confer multi-organ protection against acute ischemia/reperfusion injury in such organs as kidneys, intestine, liver, and lungs [9]. In addition, RIPC can reduce myocardial damage in patients undergoing surgery interventions [34]. In the present study, we did not find major differences in organ protection between the two groups. However, a limitation of our study is that the total number of survivors was relatively small; thus, we might not have been able to detect differences in organ protection. In the rats that survived 6 weeks, organ anatomy and function was similar between the controls and treated rats.

RIPC (brief transient episodes of ischemia separated by reperfusion) of remote tissues has been recognized as one of the most potent anti-ischemic interventions and has been extensively studied in the heart. However, in the setting of severe hemorrhagic shock, its protective effects have been investigated only to a limited extent.

Leung et al. [21] evaluated the efficacy of RIPC on organ protection after hemorrhagic shock/resuscitation in an

isoflurane-anesthetized C57Bl/6 mouse model. RIPC consisted of one instance of 10 min of left femoral artery occlusion followed by 10 min of reperfusion before the initiation of hemorrhagic shock. Control animals did not have femoral artery occlusion. Hemorrhagic shock was achieved by withdrawing blood over 15 min to lower the mean arterial blood pressure to 30 mmHg; the hypotensive period was maintained for 60 min. The animals were resuscitated with 0.9% normal saline equivalent to twice the volume of blood withdrawn. Liver and lung tissues and blood samples were collected for analysis at 1 and 2 h after resuscitation. In a second severe injury model, mice were subjected to the same procedure, but the hemorrhagic shock at 30 mmHg of mean arterial blood pressure was maintained for 2 h, and the RIPC was achieved by performing 4 cycles of alternating 5-min ischemia, followed by 5-min reperfusion. The results demonstrated that RIPC reduced levels of inflammation and injury in the liver and lung after shock/resuscitation in both mild and severe injury models. This study did not investigate survival.

In a volume-controlled hemorrhagic shock model, Hu et al. [18] tested pentobarbital-anesthetized male Sprague-Dawley rats and induced RIPC by 4 cycles of 5 min of limb ischemia followed by reperfusion for 5 min. An estimated 50% of the total blood volume was withdrawn during an interval of 60 min after performing RIPC. The mean blood pressure was maintained above 45 mmHg during the shock phase. Thirty minutes after the completion of bleeding, the shed blood was reinfused over the ensuing 30 min. These investigators found that mean blood pressure rapidly increased to near-baseline levels during the resuscitation phase and was significantly greater in the RIPC group. At 2 h after reinfusion, ejection fraction and myocardial performance index were significantly better, and the sublingual microvascular flow index and perfused vessel density were significantly greater in the RIPC group than in the control group ($p < 0.01$). At 72 h, survival in the RIPC group (7 of 7 rats) was significantly greater than that in the control group (1 of 7 survived, $p < 0.01$), and neurological deficit score was significantly better in the RIPC group than the control animals ($p < 0.01$). Our study confirms the general finding that RIPC improves acute survival but extends the previous study by showing that RIPC's benefit remains long term. Our study differs from Hu and coworkers' study in some respects: we assessed long-term survival (6 weeks), shed blood volume was less (42% versus 50%), rat mean blood pressure was lower (30 mmHg versus > 45 mmHg) during the shock phase, and in our study, rat survival was lower (50% versus 100%) in the RIPC group at 72 h after resuscitation. Other differences were anesthetic used, bleeding duration (10 min versus 60 min), and the different shock model (fixed-pressure versus fixed-volume hemorrhagic shock model).

Huang et al. [20] recently tested remote ischemic perconditioning (RIPerC, administered during the resuscitation period) and remote ischemic postconditioning (RIPostC, administered after the resuscitation period) in male rats. Hemorrhagic shock was induced by removing 45% of the estimated total blood volume, and remote ischemic conditioning was induced by four cycles of limb ischemia for 5 min followed by 5 min of reperfusion. Two hours after resuscitation, myocardial function indices such as ejection fraction and cardiac output were better in both preconditioned groups compared with a control group. Animals were monitored for 72 h before sacrifice, and survival was better in both conditioned groups compared with the control group.

The studies above support the concept that RIPC is a promising anti-ischemic intervention for acute hemorrhagic shock but do not address the long-term effects of the treatment. Our results have shown for the first time that RIPC results in long-term improvement in survival in fixed-pressure hemorrhagic shock model. In addition, we found no evidence that left ventricular dysfunction either during hypotension or after restoration of flow contributed to the shock state. We found no evidence of myocardial necrosis by biomarkers or by histology in this study and no evidence of stunned myocardium.

Hemorrhage-induced ischemia/reperfusion contributes to endothelial cell injury, impaired microcirculation, vascular permeability, and vascular leakage [35]. Ischemic preconditioning has been demonstrated in experimental models to protect endothelial function and structure and protect tissues from injury by preserving microcirculation [36–38]. Our present study supports the concept that RIPC might have reduced vascular leakage because the LVIDd was significantly higher in the RIPC group than in the control group, possibly indicating more circulating intravascular blood volume in the RIPC group than in the control group.

Vascular reactivity is greatly reduced during hemorrhagic shock. Hemorrhage-induced vascular hyporeactivity can severely interfere with the treatment of shock and may be an important cause of death [39]. RIPC can increase endothelial reactivity [40] and improve shock-induced vascular hyporeactivity [19, 41]. In the present study, we observed that early hemodynamic responses after shed blood resuscitation were improved in the RIPC group, which was indicated by the significantly higher blood pressure during the blood reinfusion phase.

The ventilation parameters used during the procedure result in normal levels of PaO_2 and $PaCO_2$ in normal rats [42]. However, in the setting of hemorrhagic shock, these or similar settings are associated with lower levels of both PaO_2 and $PaCO_2$ as previously reported [43] and as observed in our present study. Explanation for the lowering of $PaCO_2$ might be due to a mismatch of fixed lung ventilation versus reduced perfusion, resulting in relative hyperventilation. An accompanying decrease in PaO_2 might be caused by poorer relative diffusion of O_2 compared to CO_2 in a lung with reduced diffusion capacity due to lung injury from shock [44].

The technique of RIPC could be easily adapted to a scenario of a soldier facing battle as this form of therapy is

inexpensive and easy to apply. It can be thought of as a "vaccination" against the ischemia/reperfusion injury that can occur with hemorrhagic shock and could potentially reduce mortality and morbidity associated with this injury. A soldier would simply inflate a blood pressure cuff above systolic pressure for 5 min, deflate the cuff for 5 min, and then repeat this three times. This therapy could be applied daily or just prior to high-risk combat missions and therefore would be a practical and clinically relevant approach to preventative therapy. Because the therapy can be applied prophylactically prior to the soldier going into battle, RIPC as a preventative for hemorrhagic shock represents a potential therapy that is truly preconditioning rather than per- or postconditioning.

Limitations

Hemorrhagic shock-induced global organ injury depends upon the hypotensive level and the duration of shock phase. A limitation to our study is that we only investigated the protective effects of RIPC at 30 mmHg for 30 min based on a pilot time-course study. The RIPC protection under various hypotension levels and durations will require future investigation. Other limitations of the study include the concept that some aspects may be inconclusive when applied to humans. It is known that RIPC works in humans and there are several studies showing that it reduces biomarkers of necrosis in acute myocardial infarction [29]. However, whether RIPC can protect humans after blood loss is not yet known. Certainly, the results of this study suggest that it would be worth investigating, especially because soldiers could apply RIPC themselves by simply inflating and deflating a blood pressure cuff BEFORE they go onto the battlefield, i.e., this therapy could be self-administered in a prophylactic format. Whereas soldiers going into battle may represent only a small fraction of patients who lose blood, there are other situations in which prophylactic RIPC might be useful, for example, law enforcement personnel such as police or SWAT teams, who know they are going into a potentially dangerous situation. While we applied RIPC before hemorrhage, remote conditioning has also been applied at the time of injury rather than before injury; in this case, the term perconditioning has been applied. This therapy was shown to work when blood pressure cuff inflation and deflation were instituted starting in the ambulance after the onset of acute myocardial infarction [28] therapy improved myocardial salvage and long-term clinical outcome [31]. Thus, inducing a conditioning protocol after onset of injury has the potential to work in situations such as car accidents and gunshot wounds but has yet to be tested in a clinical study.

One limitation of using the rodent model is that it is difficult to do serial blood draws and blood gas measurements without altering hemodynamics. However, we were able to examine blood gases in the early resuscitation phase and draw other chemistries at 48 h. Another limitation is that we did not measure interleukin levels earlier than 48 h.

In conclusion, our data suggest that RIPC is a promising therapeutic strategy to improve long-term survival from hemorrhagic shock injury. The underlying mechanisms involved in RIPC need to be further investigated. This understanding will facilitate the development of new therapeutic strategies to help treat hemorrhagic shock.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Disclaimer Opinions, interpretations, conclusion, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

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Abstract 186: Remote Limb Ischemic Preconditioning Improves Short and Long Term Survival and Maintains Intravascular Blood Volume During Resuscitation of Hemorrhagic Shock

Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A Kloner

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Abstract

Background: We further examined the protective effects of experimental bilateral lower limb remote ischemic preconditioning (RIPC) in rats undergoing experimental hemorrhagic shock.

Methods: Sprague Dawley rats (both genders) were randomized into RIPC (n= 26) or control group (n= 27), and anesthetized with intraperitoneal ketamine/xylazine. RIPC was induced by 4 cycles of inflating small bilateral pressure cuffs to 200 mmHg around the femoral arteries for 5 minutes, followed by 5 minute deflation of the cuffs. Hemorrhagic shock was induced by withdrawing blood from the carotid artery. Target mean blood pressure of 30 mmHg was maintained for 30 minutes followed by reinfusion of shed blood within the next 30 min. Rats remained anesthetized for another 30 min before recovery; endpoints were survival at 72 hours and 6 weeks.

Results: The % of estimated total blood volume withdrawn to maintain a level of 30 mmHg was similar in the RIPC group (41.7 ± 1.0 %) and control group (41.9 ± 1.0 %). Recovery of blood pressure during the early resuscitation phase was significantly improved in the RIPC group. The diastolic internal dimension of the left ventricle (echocardiogram), which indicates circulating intravascular blood volume, was significantly larger in the RIPC group at 1 hour after initiation of shed blood reinfusion (5.8 ± 0.1 mm) compared to 5.4 ± 0.1mm in the control group (p=0.04). Left ventricular fractional shortening was comparable between RIPC (50.9 ± 1.9 %) and control group (49.6 ± 1.8 %; p=0.64) at 1 hour after initiation of resuscitation. At 48 hours after shock, BUN was within normal range in the RIPC group (17.3 ± 1.2 mg/dl); but elevated in the control group (22.0 ± 1.7 mg/dl). At 72 hours after hemorrhagic shock injury, 6 of 27 (22.2 %) rats in the control group and 13 of 26 (50 %, p = 0.047) rats in the RIPC group survived. At 6 weeks, 5 of 27 (18.5 %) rats in the control group and 13 of 26 (50 %; p = 0.021) rats in the RIPC group survived. RIPC significantly increased survival rate at both 72 hours and 6 weeks.

Conclusions: RIPC improved recovery of blood pressure and maintained more circulating intravascular blood volume in the early phase of resuscitation, improved BUN, and markedly and significantly improved short and long term survival in rats subjected to hemorrhagic shock.

Abstract 187: Effects of Anesthetic Agents on the Hemodynamic Stabilization and Long Term Survival in an Experimental Model of Hemorrhagic Shock

Wangde Dai, Jianru Shi, Juan Carreno, Sharon Hale, and Robert A Kloner

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Abstract

Background: We investigated the cardiovascular responses to acute bleeding and shed blood restoration under different anesthetic agents, and their effects on long-term survival rate after hemorrhagic shock in rats, after our initial pilot study suggested differences between anesthetics.

Methods: Sprague Dawley rats (both genders) were randomized to receive either intraperitoneal ketamine/xylazine (K/X, 90 mg/kg and 10 mg/kg; n=12), or isoflurane (5% isoflurane induction and 2% maintenance in room air; n=12) for anesthesia. Blood was withdrawn from the carotid artery to maintain mean arterial blood pressure (MBP) at 30 mm Hg for one hour, followed by 30 min of resuscitation with shed blood. Rats remained anesthetized for another 30 min before they were allowed to recovery and survive for 6 weeks. Hemodynamics were monitored during the surgical procedure.

Results: During the shock phase, the total withdrawn blood volume (expressed as % of estimated total blood volume) to maintain MBP at 30 mmHg was significantly higher in the isoflurane group (51 ± 1.5 %) compared to the K/X group (45.3 ± 1.8 %; p=0.023). The diastolic internal dimension of the left ventricle, which indicated circulating intravascular blood volume, was significantly larger in the isoflurane group at the end of 1 hour of the shock phase (4.5 ± 0.2 mm compared to 3.5 ± 0.2 mm in K/X group; p=0.0003) and at 1 hour after initiation of shed blood reinfusion (6.3 ± 0.2 mm compared to 5.3 ± 0.3 in K/X group; p=0.014). Recovery of blood pressure during the resuscitation phase was significantly improved in the isoflurane group compared to the K/X group. The survival rate at 6 weeks was 1 of 12 (8.3%) in rats receiving K/X and 10 of 12 (83.3%) in rats receiving isoflurane ($p < 0.001$). Histology demonstrated brain infarction in the 1 surviving rat receiving K/X; no brain infarction in the 10 surviving rats that received isoflurane at 6 weeks. No infarction was detected in heart, lung, liver or kidneys in all surviving rats.

Conclusions: These results suggest that isoflurane stabilizes the cardiovascular response to acute blood lose and benefits the perfusion of tissue, which resulted in significantly higher long term survival rate and improved blood pressure response to resuscitation, without end-organ infarction.

Abstract 191: Effects of Anesthetic Agents on Blood Parameters in Rats With Acute Hemorrhagic Shock

Jianru Shi, Wangde Dai, Juan Carreno, Sharon L Hale, and Robert A Kloner

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Abstract

Background: Recent studies in our laboratory indicate that isoflurane (ISO) has protective properties including improved survival in rats with hemorrhagic shock compared to ketamine and xylazine (K/X) anesthesia. The effects of these two anesthetic agents upon blood counts, gases and chemistries in the setting of hemorrhagic shock is unknown. The purpose of the present study was to examine the effects of these two commonly used anesthetic regimens on blood parameters in rats with acute hemorrhagic shock.

Methods and Results: Sprague Dawley rats (both genders) were anesthetized with either intraperitoneal K/X (90mg/kg and 10mg/kg; n=12) or with isoflurane (5% isoflurane induction and 2% maintenance in room air; n=12). Rats were intubated and ventilated with room air. After heparinization, hemorrhagic shock was induced by withdrawing blood to a fixed mean blood pressure of 30 mmHg for one hour and then shed blood was reinfused. Arterial blood samples were collected at 1 hour after resuscitation with shed blood. We found that K/X was associated with lower PH and lower level of standard bicarbonate concentration (SBC) and oxygen saturation (SO_2%) and more negative base excess; and had a significantly elevated anion gap, potassium, sodium and chloride levels compared to isoflurane (Table). Platelet counts were preserved and there was less elevation of white blood cell (WBC) in ISO (Table). There were no significant differences in PO_2, PCO_2, hematocrit, hemoglobin, glucose and lactate levels between the two types of anesthesia.

Conclusions: The anesthesia influenced the levels of blood counts, gases and chemistries in rats with acute hemorrhagic shock, favoring ISO over K/X. Blood parameters remained essentially normal in ISO group, which may help explain the protective role of ISO in hemorrhagic shock.

Table: Comparison of blood parameters between K/X and ISO group. Data are presented as mean ± SE. p<0.05 vs ISO.

	K/X (n=12)	ISO (n=12)	P value
pH	7.32 ± 0.04	7.47 ± 0.02	0.0025
SBC (mmol/L)	18.22 ± 1.08	23.15 ± 0.87	0.0019
Base Excess (mmol/L)	-7.80 ± 1.45	-1.57 ± 1.02	0.0023
SO2 %	91.91 ± 1.66	96.57 ± 0.40	0.0177
AnionGap (mmol/L)	12.8 ± 1.07	8.81 ± 0.98	0.0119
Sodium (mmol/L)	137.82 ± 1.06	133.62 ± 0.71	0.0038
Potassium (mmol/L)	5.98 ±0.31	4.82 ± 0.21	0.0064
Chloride (mmol/L)	114.33 ± 1.38	109.23 ± 0.85	0.0056
Platelet counts (K/uL)	441.08 ± 51.00	597.42 ± 34.59	0.0199
WBC (K/ul)	9.88 ± 0.80	7.23 ± 0.82	0.0304

Therapeutic hypothermia alone and in combination with preconditioning improves long term survival during resuscitation of hemorrhagic shock

Wangde Dai, Jianru Shi, Juan Carreno, and Robert A. Kloner

HMRI Cardiovascular Research Institute, Huntington Medical Research Institutes, 686 South Fair Oaks Avenues, Pasadena, CA 91105, and Division of Cardiovascular Medicine of the Keck School of Medicine, University of Southern California, Los Angeles, California 90017-2395.

Background: We investigated the effects of hypothermia treatment alone and in combination with experimental bilateral lower limb remote ischemic preconditioning (RIPC) in rats undergoing experimental hemorrhagic shock. Previously we showed that RIPC alone improved survival.

Methods: In the hypothermia alone study, adult Sprague Dawley rats (both genders) were randomized to hypothermia (n=16) or control group (n=15); in a combination study, rats were randomized to hypothermia plus RIPC (n=11) or control group (n=10). Rats were anesthetized with intraperitoneal ketamine/xylazine. Therapeutic hypothermia (Thermosuit) was started at 5 minutes after mean blood pressure (MBP) reached 30 mmHg. Core temperature was maintained at ~ 32 °C until blood volume was fully restored. RIPC was induced by 4 cycles of inflating small bilateral pressure cuffs to 200 mmHg around the femoral arteries for 5 minutes, followed by 5 minute deflation of the cuffs prior to hemorrhagic shock. In the control group, body temperature was maintained at 37 °C during the procedure. Hemorrhagic shock was induced by withdrawing blood from the carotid artery. Target mean blood pressure of 30 mmHg was maintained for 30 minutes followed by reinfusion of shed blood within the next 30 min. Rats were allowed recovery and the primary endpoint was survival rate at 6 weeks.

Results: In the setting of hypothermia alone, 4 of 15 (26.7 %) rats in the control group and 11 of 16 (68.8 %; p = 0.032) rats in the hypothermia group survived at 6 weeks. In the combination study, 1 of 10 (10 %) rats in the control group and 7 of 11 (63.6 %; p = 0.024) rats in the hypothermia plus RIPC group survived at 6 weeks. Kaplan Meier Survival Curves are shown in Fig1.

Conclusions: Hypothermia alone and combination with RIPC significantly improved long term survival in rats subjected to hemorrhagic shock. Hypothermia and RIPC did not show survival rates greater than hypothermia alone.

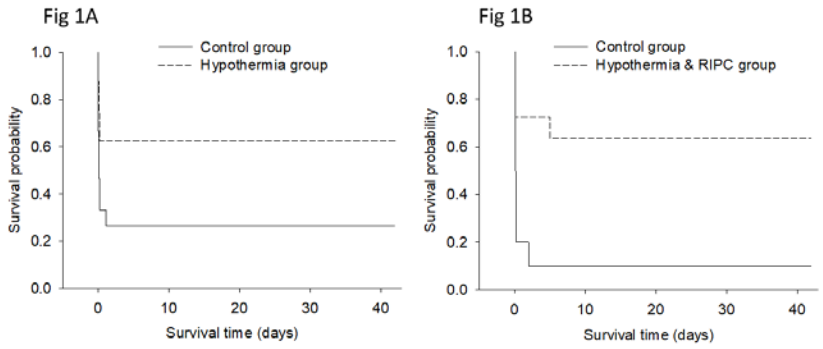

Fig 1: Kaplan Meier Survival Curve of the post-resuscitation survival rate. A: Treatment with hypothermia only and control group study; B: Treatment with hypothermia plus RIPC and control group study.

Effects of Therapeutic Hypothermia on Blood Parameters in Rats with Acute Hemorrhagic Shock

Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner

HMRI Cardiovascular Research Institute, Huntington Medical Research Institutes, 686 South Fair Oaks Avenues, Pasadena, CA 91105, and Division of Cardiovascular Medicine of the Keck School of Medicine, University of Southern California, Los Angeles, CA 90017

Background: Our research group recently observed that therapeutic hypothermia (TH) compared with normothermia improved long-term survival in an experimental model of hemorrhagic shock. The effect of TH on blood counts, blood gases and chemistries during the early phase of recovery from hemorrhagic shock are unknown. Therefore, the purpose of the present study was to examine the effects of TH on blood parameters in the early phase of resuscitation from hemorrhagic shock.
Methods and results: Sprague Dawley rats (both genders) were randomly assigned to TH (n= 16) or normothermia group (n= 15). Rats were anesthetized with intraperitoneal ketamine and xylazine. After heparinizing, hemorrhagic shock was induced by withdrawing blood to a fixed mean blood pressure (MBP) of 30 mmHg for 30 minutes and then shed blood was reinfused. TH was started 5 minutes after MBP reached 30 mmHg. Core temperature was maintained at ~ 32 °C until blood volume was fully restored, after which the rats were allowed to warm back to normal temperature. In the normothermia group, body temperature was maintained at ~ 37°C. Arterial blood samples were collected 1 hour after resuscitation with shed blood. We found that pO_2 (partial pressure of oxygen) was significantly higher in TH group versus the normothermic group. The rats in normothermic group had significantly elevated potassium, chloride and lactate levels and more negative base excess compared to rats that in TH group (Table). The neutrophil was lower in the TH group; the lymphocyte (%) was higher in the TH group. There were no significantly differences in pH, pCO_2, sodium, calcium or glucose between the normothermia and TH groups.
Conclusions: pO_2 remained normal and levels of potassium, chloride, lactate and neutrophil were lower in TH group. These results may contribute to the protective effect of TH during hemorrhagic shock.

Table: Comparison of blood parameters between normothermia and therapeutic hypothermia (TH) group. Data are presented as mean ± SE. p<0.05 vs control.

	Normothermia (n=15)	TH (n=16)	P value
pO2 (mmHg)	81.6 ± 4.8	117.8 ± 9.9	0.004
Potassium (mmol/L)	5.4 ± 0.2	4.1 ± 0.1	<0.0001
Chloride (mmol/L)	112.3 ± 0.6	110.4 ± 0.5	0.024
Base Excess (mmol/L)	-6.8 ± 0.5	-5.6 ± 0.3	0.043
Lactate (mmol/L)	1.8 ± 0.2	1.2 ± 0.2	0.028
Neutrophil (%)	22.9 ± 1.6	16.1 ± 1.4	0.003
Neutrophil (/uL)	2223.7 ± 248.7	1756.6 ± 219.4	0.170
Lymphocyte (%)	74.5 ± 1.6	81.7 ± 1.4	0.002
Lymphocyte (/uL)	7212.9 ± 562.4	8990.1 ± 713.1	0.061

Therapeutic Hypothermia Alone and Combination with Preconditioning Blunt Inflammation in Experimental Hemorrhagic Shock

Jianru Shi, Wangde Dai, Juan Carreno, Sharon L. Hale, Robert A. Kloner

HMRI Cardiovascular Research Institute, Huntington Medical Research Institutes, 686 South Fair Oaks Avenues, Pasadena, CA 91105, and Division of Cardiovascular Medicine of the Keck School of Medicine, University of Southern California, Los Angeles, CA 90017

Background: Recent studies by our group indicate that preconditioning, therapeutic hypothermia (TH) and TH combined with preconditioning improved long-term survival during resuscitation of hemorrhagic shock. The neutrophil-to-lymphocyte ratio (NLR) is a marker of inflammation associated with increased mortality in patients with severe hemorrhage shock. The aim of this study is to evaluate the effects of these three therapies on NLR level in rats with acute hemorrhagic shock.

Methods and results: In the preconditioning study, Sprague Dawley rats (both genders) were randomized to preconditioning (n=26) or control group (n=27); in the hypothermia study, rats were randomized to TH (n=16) or control group (n=15); in a combination study, rats were randomized to TH plus preconditioning (n=11) or control group (n=10). Rats were anesthetized with intraperitoneal Ketamine and xylazine. After heparinizing, hemorrhagic shock was induced by withdrawing blood to a fixed mean blood pressure (MBP) of 30 mmHg for 30 minutes and then shed blood was reinfused. Preconditioning was induced by 4 cycles of inflating small cuffs around the femoral arteries to 200 mmHg for 5 minutes, followed by 5-minute deflation of the cuffs prior to hemorrhagic shock. TH started at 5 minutes after MBP reached 30 mmHg. Core temperature was maintained at ~32 °C until blood volume was fully restored. In the control group, body temperature was maintained at ~ 37°C. Arterial blood samples were collected 1 hour after resuscitation. The NLR is an easily accessible biomarker, which is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. The NLR was significantly lower in TH group (0.20 ± 0.02) compared with the control group (0.32 ± 0.03; p=0.003). Similarly, the NLR level was significantly decreased in TH plus preconditioning group (0.19 ± 0.02) versus the control group (0.33 ± 0.02; p= 0.001). There was no difference in NLR level between the preconditioning group (0.41 ± 0.04) and the control group (0.41 ± 0.04; p=0.984).

Conclusions: NLR is widely recognized inflammation marker associated with poor prognosis in severe hemorrhagic shock. TH alone and TH combined with preconditioning blunt the inflammation by decreasing the NLR level in experimental hemorrhagic shock.