The Evolution of Chemokine Release Supports a Bimodal Mechanism of Spinal Cord Ischemia and Reperfusion Injury

Phillip D. Smith, MD; Ferenc Puskas, MD, PhD; Xianzhong Meng, MD, PhD; Joon H. Lee, MD; Joseph C. Cleveland, Jr, MD; Michael J. Weyant, MD; David A. Fullerton, MD; T. Brett Reece, MD

Background—Paraplegia remains a devastating complication of thoracic aortic surgery. The mechanism of the antecedent spinal cord ischemia and reperfusion injury (IR) remains poorly described. IR involves 2 injuries, an initial ischemic insult and subsequent inflammatory amplification of the injury. This mechanism is consistent with the clinical phenomenon of delayed onset paraplegia. This study sought to characterize the inflammatory response in the spinal cord after IR and hypothesized that this would support a bimodal mechanism of injury.

Methods and Results—Male C57Bl/6 mice were subjected to 5 minutes of aortic arch and left subclavian occlusion with subsequent reperfusion to generate spinal cord ischemia. Functional outcomes were scored at 12-hour intervals. Spinal cords were harvested after 0, 6, 12, 18, 24, 36, and 48 hours of reperfusion. Cytokine levels were analyzed using a mouse magnetic bead–based multiplex immunoassay. Inflammatory chemokine concentrations (interleukin [IL]-1β, IL-6, keratinocyte-derived cytokine, macrophage inflammatory protein-1α, monocyte chemotactic protein-1, RANTES, and tumor necrosis factor-α) peaked at 6 hours and 36 to 48 hours after reperfusion. Functional scores reflected initial gain in function with subsequent decline, inversely proportional to cytokine levels. Immunofluorescent staining demonstrated microglia activation at 12 and 48 hours.

Conclusions—Spinal cord ischemia and reperfusion injury occurs in 2 phases, correlating to increases in inflammatory chemokines release and microglial activation. These observations chronologically parallel the too-common clinical syndrome of delayed-onset paraplegia. Understanding the molecular pathogenesis of this injury may allow future intervention to prevent this devastating complication. (Circulation. 2012;126[suppl 1]:S110–S117.)

Key Words: aorta ■ reperfusion injury ■ stroke prevention

Spinal cord ischemia and reperfusion injury remains a devastating complication of thoracic aortic intervention, both open and endovascular. Distal aortic perfusion and cerebrospinal fluid drainage have significantly improved neurological complication rates. Other techniques, such as intercostal artery reimplantation, evoked potential monitoring, epidural cooling, and hypothermic circulatory arrest also appear to modestly reduce risk of spinal cord injury. Unfortunately, no pharmacological adjuncts have proven clinically efficacious in attenuating this injury. Moreover, once the injury has clinically manifested, no pharmacological or surgical intervention (other than attempts to modify parenchymal perfusion) is currently available.

The phenomenon of delayed neurological deficits after these procedures highlights the complexity of the injury. Progression of the injury and a delay in symptom onset suggest that the pathological mechanisms involve not only the initial ischemic event, but ongoing molecular insults during the reperfusion phase. Some of the first characterizations of spinal cord injury after aortic procedures reported a total incidence of 15% to 20%, with slightly more than half of those occurring in a delayed fashion. Recent studies from high-volume centers suggest significant improvement in outcomes with an incidence of spinal cord injury around 3%, but more than 80% of these presenting with delayed onset of symptoms. Furthermore, delayed paraplegia is not always reversible. Thus, the pathogenesis of this delayed injury has become increasingly clinically relevant.

Ischemia and reperfusion injury in other organ systems has been much more extensively studied than in the spinal cord. In these other systems, the inflammatory molecular milieu after ischemia and reperfusion injury appears to play a role in the progression of end organ damage. Transient myocardial ischemia, or ischemia and reperfusion (IR), activates the innate immune system to stimulate inflammatory signaling cascades that ultimately lead to myocardial injury. Inflammatory cytokine expression after acute cerebral ischemia is enhanced and is positively correlated with infarct volume. Furthermore, many authors postulate that resident leukocytes
play a significant role in the inflammation of reperfusion. These observations in other organ systems led us to believe that an analogous inflammatory milieu, with activated microglia playing the role of resident leukocyte, contributes to the progression of neurological deficits after spinal cord ischemia and reperfusion. We aimed to characterize the evolution of the inflammatory response in the spinal cord after aortic occlusion.

Methods

Animal Procedures
The Animal Care and Use Committee at the University of Colorado at Denver Health Sciences Center approved all experiments, and this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (www.nap.edu/catalog/5140.html). C57Bl/6 male mice were obtained from Jackson Laboratories. Mice between 12 and 20 weeks of age were used for experimentation. Using 2% isoflurane anesthesia, a cervicothoracic approach was used to expose the aortic arch as previously described.13–16 The aortic arch was cross-clamped between the left common carotid artery and the left subclavian artery under direct visualization. Normothermia was maintained using a temperature controlled operating bed. Ischemia continued for 4 minutes, after which clamps were removed. An additional clamp was placed on the left subclavian artery. Disruption of distal aortic flow was confirmed using a laser Doppler blood flow monitor (Moor Instruments, Devon, United Kingdom) to achieve a greater than 90% decrease in distal aortic flow measured at the femoral artery. Sham mice had the aortic arch exposed through the same procedure but no aortic cross-clamping. Animals were euthanized at 0, 6, 12, 18, 24, 36, and 48 hours after reperfusion, and their spinal cords were harvested for analysis. Four animals were included at each of the 7 time points. Four additional sham time points (0, 6, 24, and 48 hours) were chosen for comparison with four animals in each of these groups.

Functional Locomotor Scores
The Basso mouse scale for locomotion was used to quantify hind limb function in mice after ischemia, which ranges from a score of 0 for complete paraplegia to a score of 9 for normal function (Table).17 Function was scored at 12, 24, 36, and 48 hours after reperfusion.

Histological Analysis
T-10 through L-3 vertebral columns were removed en bloc, and the spinal cord was harvested by forcefully injecting phosphate-buffered saline (PBS, pH 7.4) into the spinal cord. Spinal cords were preserved in 10% formalin for at least 24 hours before paraffin embedding, sectioning, and hematoxylin and eosin staining.

Immunofluorescent Iba-1 Staining
For immunofluorescent staining, mice were first perfused with 0.9% normal saline, followed by 4% paraformaldehyde. T-10 through L-3 vertebral columns were removed en bloc, and the spinal cord was harvested by forcefully injecting PBS (pH 7.4) into the spinal cord. Samples were then cut in cross-sections and placed in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA) at −80°C.

Cryosections (16 μm thick) were cut with a cryostat (IEC Minotome Plus; International Equipment Co, Needham Heights, MA) and collected on poly-l-lysine–coated slides. Sections were treated with a mixture of 70% acetone and 30% methanol for 10 minutes and then fixed with 4% paraformaldehyde for 10 minutes. Sections were washed with PBS, blocked with 10% normal serum for 30 minutes, and incubated overnight with a polyclonal rabbit anti-mouse ionized calcium binding adapter molecule 1 (Iba-1) antibody (Wako Chemicals USA, Richmond, VA) at a 1:150 dilution of PBS containing 1% bovine serum albumin antibody. After washing with PBS, sections were incubated with Cy3-conjugated matched IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) at 1:100 dilution with PBS containing 1% bovine serum albumin. To assess specificity, adjacent sections were incubated with non–immune-matched IgG (5 μg/mL in PBS containing 1% bovine serum albumin) and otherwise processed identically. Primary incubation was performed at 4°C overnight, and all other incubations were performed at room temperature. To stain nuclei, bis-benzimide was used (4′,6-diamidino-2-phenylindole, imaged on the blue channel), and wheat-germ agglutinin was used to stain cell membranes (labeled with Alexa 488 and imaged on the green channel). Iba-1 was imaged using the red (Cy3) channel. Microscopic observation and photography were performed with a Leica DMRXA confocal microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Table. Mouse Basso Motor Score for Hind Limb Function

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No ankle movement</td>
</tr>
<tr>
<td>1</td>
<td>Slight ankle movement</td>
</tr>
<tr>
<td>2</td>
<td>Extensive ankle movement</td>
</tr>
<tr>
<td>3</td>
<td>Plantar placement of the paw with or without weight support, or occasional, frequent—or—consistent dorsal stepping but no plantar stepping</td>
</tr>
<tr>
<td>4</td>
<td>Occasional plantar stepping</td>
</tr>
<tr>
<td>5</td>
<td>Frequent or consistent plantar stepping, no coordination—or—frequent or consistent plantar stepping, some coordination, paws rotated at initial contact and lift-off</td>
</tr>
<tr>
<td>6</td>
<td>Frequent or consistent plantar stepping, some coordination, paws parallel at initial contact—or—frequent or consistent plantar stepping, mostly coordinated, paws rotated at initial contact and lift-off</td>
</tr>
<tr>
<td>7</td>
<td>Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift-off (P/R)—or—frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift-off (P/P), and severe trunk instability</td>
</tr>
<tr>
<td>8</td>
<td>Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift-off (P/P), and mild trunk instability—or—frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift-off (P/P), and normal trunk stability and tail down or up and down</td>
</tr>
<tr>
<td>9</td>
<td>Frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift-off (P/P), and normal trunk stability and tail always up</td>
</tr>
</tbody>
</table>

Sample Preparation and Cytokine Quantification
Each specimen intended for cytokine analysis (n=4 for each time point) was flash-frozen using 2-methylbutane (Fisher Scientific, Fair Lawn, NJ) and dry ice and stored immediately in −80°C. Samples were thawed to 4°C, weighed, homogenized using 10 μL of ethylenediaminetetraacetic acid-free, complete lysis-M buffer (Roche Diagnostics Corporation, Indianapolis, IN) per 1 mg of specimen. Each sample was then sonicated for 30 seconds to achieve cell demembranization and centrifuged (12 000g at 4°C) for 20 minutes. Protein quantification was performed for each sample using a Coomassie Plus (Bradford) assay kit according to manufacturer’s instructions (Pierce Protein Research Products, Thermo Scientific, Rockford, IL). Bovine serum albumin was used as a standard. Absorbance of standards and samples were determined spectrophotometrically at 595 nm, using a microplate reader (Bio-Rad Laboratories, Inc, Hercules, CA). Results were plotted against the linear portion of the standard curve, and the protein concentration of each sample was expressed as micrograms per milliliter of sample.

A mouse cytokine magnetic bead-based multiplex assay (Bio-Rad Laboratories, Inc, Hercules, CA) was used to quantitatively measure the expression of the following 23 murine cytokines: interferon-
(IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), keratinocyte-derived factor (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T expressed and secreted protein (RANTES), and tumor necrosis factor-α (TNF-α). Dilutions of 1:10, using our lysis buffer, were prepared and stored at −80°C. Samples and standards were prepared according to manufacturer’s instructions with each standard run in duplicate. Cytokine expression was determined using the Luminex 100 multiplex assay plate reader (Luminex Corporation, Austin, TX), and protein concentration was expressed as picograms per milliliter.

Statistical Analysis
For multiplex assay results, the raw data were normalized to the total protein concentration of each specimen, and the normalized data were used to perform statistical analysis.

Data are presented as mean±SEM. Statistical analysis was performed using ANOVA with Tukey-Kramer post hoc analysis for pairwise comparisons (StatView by SAS Institute Inc, Cary, NC). For all statistical comparisons, a probability value <0.05 was considered significant.

Results
Functional Outcomes
Functional hind limb outcomes were similar to that observed previously. After 4 minutes of spinal cord ischemia and subsequent reperfusion, all mice had some initial motor deficit. Over the following 48-hour observation period, mice regained some neurological function in the first 24 hours, with a subsequent decline in function up to 48 hours (Figure 1).

Cytokine Expression
Of the 23 cytokines analyzed, the chemokines IL-1β, IL-6, KC (murine equivalent of human IL-8), MIP-1α, MCP-1, RANTES, and TNF-α showed a biphasic response. These cytokines were selected from the panel of 23 because of their biological significance as chemotactic factors (making them chemokines, by definition). Of the 7 chemokines selected, ANOVA among the means demonstrated significant differences between groups. F values were significant with probability values ≥2.9% in each of these 7 chemokines. Tukey-Kramer post hoc analysis revealed significant differences among pairwise comparisons.

Overall, chemokine production peaked at 6 and 36 to 48 hours (Figure 2 and Figure 3). IL-1β expression was significantly increased from baseline expression (time point 0) at 6 and 36 hours into reperfusion. Expression at 6 and 36 hours was also increased compared with 18 hours, signifying a biphasic response to reperfusion. MIP-1α showed an even more exaggerated biphasic response. Expression of MIP-1α was increased from baseline at 6 and 36 hours. In addition, increased expression at 6 and 36 hours was significant compared with all other time points (0, 12, 18, 24, and 48 hours). Expression of these 2 cytokines at 6 and 36 hours was also increased from shams at most time points (specific pairwise comparisons given in Online Data Supplement Table I).

IL-6 and MCP-1 peaked at 36 hours, with no significant increase in expression at other time points. IL-6 was significantly increased compared with all other time points, including sham time points. MCP-1 was significantly increased compared with all time points except 48 hours of reperfusion.
RANTES and KC expression also peaked at 6 and 36 hours; however, the 6-hour peak was not marked enough to meet statistical significance using the methods described. The peak in RANTES expression at 36 hours was statistically increased over all other time points. KC expression peaked at 36 hours, meeting statistical significance when compared with 18 hours of reperfusion.

TNF-α showed trends toward a biphasic, early and late, peak in expression. However, this did not meet statistical significance using the methods described. Significant increases are detailed in Online Data Supplement Table I.

**Histology**

Hematoxylin and eosin–stained images demonstrated increasing tissue damage consistent with the functional data observed. By 48 hours of reperfusion, motor neurons demonstrate collapsed nuclei with dense chromatin bodies. Mitochondrial and endoplasmic reticulum condensation is reflected by shrunken cytoplasm. Increased vacuolization is also seen at 24 hours (Figure 4). All of these changes are consistent with progressive apoptosis of anterior horn motor neurons. Chemotaxis of inflammatory cells is evident by 24 and 48 hours of reperfusion.

**Iba1 Immunofluorescence**

Iba1 immunofluorescence was used to determine the relative activation and populations of microglia after spinal cord ischemia and reperfusion. Normal spinal cord tissue shows almost no microglial activation and only scant staining with Iba1 (Figure 5). Iba1 immunofluorescence was increased at 12 hours and at 48 hours of reperfusion, with relatively little activation present at 24 hours and with no ischemia.

**Discussion**

The dearth of evidence for the pathophysiologic mechanisms of spinal cord ischemia and reperfusion injury prompted this study. Inflammation, although ubiquitous in all tissue injury, has a tissue specific role, contributing variably in magnitude and mechanism to the overall effect of ischemia and reperfusion. The present studies’ characterization of the inflammatory response after spinal cord IR suggests a biphasic molecular response to the injury. In addition, the present study correlates the activation of microglia with both the biphasic chemokine response and the functional motor neuron injury after spinal cord IR.

Cerebral damage and repair after ischemia and reperfusion is known to be modulated by soluble proinflammatory mediators such as cytokines, chemokines, adhesion molecules, and matrix metalloproteinases. Chemokines, a subclass of cytokines inducing leukocyte chemotaxis, appear to be of particular importance in regulating these injuries. After spinal cord IR, early chemokine production by local cellular populations may result in chemotaxis of more inflammatory cells that ultimately lead to functional motor deficits. Chemokine production by this second wave of inflammatory cells further amplifies the response and results in irreversible neurological injury. It is the accumulation of these molecular insults that reaches a threshold and thus results in clinically apparent neurological injury.

IL-1β, IL-6, KC (murine equivalent of human IL-8), MIP-1α, MCP-1, RANTES, and TNF-α are known to be important mediators after cerebral ischemia. Perhaps not surprisingly, our results implicate them in the pathogenesis of spinal cord IR as well.
IL-1β expression results in a broad spectrum of proinflammatory effects that have been heavily implicated in disease states associated with immune dysregulation, ranging from rheumatoid arthritis to myocardial infarction. In cerebral IR, IL-1β production is associated with both early ischemic and late reperfusion injury. At both phases in the brain, production appears dependent on activated microglia. The immediate and delayed expression and secretion of IL-1β observed in our study, as well as the activation of microglia at corresponding time points, strongly implicates these cells in the pathogenesis of spinal cord IR.

MIP-1α is also an important chemotactic protein that induces inflammation in the central nervous system after initial insult. MIP-1α promotes monocyte and neutrophil infiltration and has been shown to colocalize with activated microglia. Early secretion by activated microglia after spinal cord IR, as implicated in our study, may significantly contribute to the subsequent inflammatory cascade that results in delayed motor neuron injury. KC (murine IL-8 homologue) is yet another chemokine known to be secreted by activated microglia after inflammatory insults. KC pro-

Figure 3. Chemokine concentrations of interleukin IL-1β (A), IL-6 (B), keratinocyte-derived factor (KC) (C), monocyte chemotactic protein-1 (MCP-1) (D), macrophage inflammatory protein (MIP)-1α (E), RANTES (F), and tumor necrosis factor-α (TNF-α) (G) in spinal cord homogenates after ischemia and reperfusion (IR). Mice undergoing IR and sham operations are shown. IL-1β and MIP-1α show a peak in expression at 6 and 36 hours. MCP-1 and IL-6 peak at 36 hours. RANTES and KC expression increased at 6 hours and again up to 48 hours. TNF-α expression increased after 24 hours until 48 hours. Data are presented as mean±SEM. n=4 at each time point.
duction early in the reperfusion phase of spinal cord IR further implicates the role of resident microglia cells.

The increased expression of IL-6, MCP-1, RANTES, and TNF-α observed in this study also correlates with microglia activation after 48 hours of reperfusion. However, the specific contribution of each chemokine produced early, late, or in a bimodal distribution remains to be determined and was not the focus of this study.

The concept of resident inflammatory cells inciting a prolonged response and clinical injury is not new. Kupffer cells in the liver and alveolar macrophages in the lung induce the proinflammatory signaling cascades responsible for IR injury after orthotopic transplantation. In addition, a growing body of evidence has suggested a role of resident microglia in the evolution of ischemic cerebral injury. Our data support an analogous role of microglia in the evolution of spinal cord injury after IR.

Previous studies have shown decreases in parenchymal perfusion secondary to decreased collateral network pressure to be a significant factor determining spinal cord injury after surgery. This concept readily explains many of the known predisposing factors to spinal cord injury (ie, hypotension, increased cerebrospinal fluid pressure). The present study is complementary to this concept in that decreased parenchymal perfusion (or decreases in collateral networks) incites the subsequent inflammatory response. To date, all strategies to attenuate spinal cord injury are aimed at increasing parenchymal perfusion in some form. The significance of the present study is that it identifies specific immune mechanisms that can be targeted.

The identification of these molecular and cellular pathways, and their significance in the mechanism of spinal cord injury, is noteworthy. These early findings may eventually be used to tailor therapeutic interventions to the cellular and molecular culprits responsible for the injury. Some of these investigations have already been reported assuming the role of these inflammatory mechanisms in the injury progression. These data confirm that the inflammatory molecules are potential targets for injury attenuation. Etanercept, a TNF-α antagonist, has been shown to be neuroprotective in murine models of spinal cord trauma. IL-1 receptor antagonist has also proven beneficial in attenuating murine spinal cord compression injury. However, the true significance of our results lies in the implication of a tissue specific cell population in the spinal cord responsible for inciting the inflammatory cascade resulting in delayed neurological injury. This phenomenon of local inflammation, as opposed to a systemic response, has been reported by other investigators and implicated in delayed neurological injury after spinal cord IR. Biphasic chemokine production also has precedent in the literature after renal IR has been reported and is implicated in different stages of inflammation after renal transplantation. By targeting the microglia cellular populations, interventions can be designed specific to spinal cord without the deleterious and immunosuppressive effects of systemically inhibiting cytokine production. This observation has important clinical implications. A delayed inflammatory response incited by resident cells might explain the phenomenon of late onset neurological complications. In addition, it defines a window in which interventions may allow attenuation of this response and thus an attenuation of the neurological injury.

The present study does have limitations, however. First, the number of animals included in each group may be limited relative to the breadth of inflammatory mediators studied. Several of the cytokines and chemokines examined may prove to have a significant increase in expression that our study was not powered to detect. In addition, we chose to measure 23 different cytokines and selected the biologically significant interactions for further detailed analysis. Inherent in doing so, we inserted our own bias into the mechanisms of
this injury into our study. In addition, our experimental design established a temporal and associative relationship between microglia activation and chemokine production, but not a causal one. The model itself is also a limitation to some extent. It does not allow for spinal fluid drainage or close control of postoperative mean arterial pressure as it might in the clinical scenario. However, the delayed fashion of the deficit certainly parallels that seen in the procedures of interest despite what may appear to be a short ischemic insult. The model does provide some molecular insight into this injury that is not clinical available. Future studies will better elucidate the nature of the relationship between the inflammation and the neuronal injury so that it might be attenuated.

In summary, the results of the present study define 2 periods of chemokine production after spinal cord ischemia and reperfusion. Early expression is likely due to resident microglia cells in the spinal cord. Later production may be due to recruitment of systemic inflammatory cells to the tissue. These observations will allow future studies to tailor therapies specific to the spinal cord injury after ischemia and reperfusion to optimally preserve functional outcomes after aortic interventions.

Acknowledgments
We would like to acknowledge and thank the Thoracic Surgery Foundation for Research and Education for their financial support in this project.

Disclosures
None.

References
1. Safi HJ, Miller CC III, Huynh TT, Estrera AL, Porat EE, Winnekvist AN, Allen BS, Hassoun HT, Moore FA. Distal aortic perfusion and cerebrospinal fluid drainage for thoracoabdominal and descending
The Evolution of Chemokine Release Supports a Bimodal Mechanism of Spinal Cord Ischemia and Reperfusion Injury
Phillip D. Smith, Ferenc Puskas, Xianzhong Meng, Joon H. Lee, Joseph C. Cleveland, Jr, Michael J. Weyant, David A. Fullerton and T. Brett Reece

Circulation. 2012;126:S110-S117
doi: 10.1161/CIRCULATIONAHA.111.080275

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/126/11_suppl_1/S110

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/09/11/126.11_suppl_1.S110.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
<table>
<thead>
<tr>
<th>Pairwise Timepoint</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>KC</th>
<th>MCP-1</th>
<th>MIP-1α</th>
<th>RANTES</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>0-12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>0-18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>0-24</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>0-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>6-12</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>6-18</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>6-24</td>
<td>↑</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>6-36</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>6-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>12-18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>12-24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>12-36</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>12-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>18-24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>18-36</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>18-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>24-36</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>24-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>36-48</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>6-sham6</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>24-sham24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>48-sham48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
</tbody>
</table>

Supplementary Table 1: Pairwise comparisons evaluated using Tukey-Kramer post hoc analysis. Arrows indicate a significance level of 5% and the direction of change.