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Title:

Semaphorin 3A expression following intestinal ischemia/reperfusion injury in Sox10-Venus mice.

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Abstract

PURPOSE: Semaphorin 3A (Sema3A) is a protein secreted during development of the nervous system that plays an important role in neuronal pathophysiology. However, there is no known correlation between Sema3A and intestinal ischemia/reperfusion (I/R) injury. We assessed Sema3A expression and distribution in relation to enteric nervous system (ENS) damage seen after intestinal I/R injury in Sox10-Venus mice.

METHODS: Intestinal I/R injury was induced by vascular occlusion for 3 hours. Ileal specimens were harvested 0, 3, 12, 24, 48, and 96 hours after reperfusion. Stereoscopic microscopy and fluorescence microscopy were used to assess sox10-Venus^{+} cells and PGP9.5^{+} cells.

RESULTS: By 3 hours after reperfusion, Sema3A expression had increased to a maximum and Sox10-Venus^{+} cells had faded to a minimum in harvested ileal segments. Both differences were statistically significant. By 96 hours after reperfusion, both Sema3A and Sox10-Venus^{+} cell fluorescence had reverted to original levels. Hematoxylin and eosin staining identified histologic damage mimicking Sema3A expression while PGP9.5^{+} cell response was minimal.

CONCLUSION: We are the first to demonstrate a correlation between Sema3A expression and ENS damage following intestinal I/R in Sox10-Venus mice.

Keywords: ischemia/reperfusion injury, semaphorin 3A, Sox10-Venus/C57BL GFP transgenic mice

Introduction

Semaphorin 3A (Sema3A) is a protein secreted during the development of the nervous system that acts as a cue for axon induction [1] and growth of peripheral axons by regulating growth cone collapse. Sema3A is also expressed by activated T cells and dendritic cells and is known to induce apoptosis in immune cells as well as be involved in the pathology of inflammatory diseases [1]. Recently, Sema3A was assessed after acute renal ischemia/reperfusion injury (I/R) and considered to play a pathogenic role in protein mediated apoptosis of renal epithelial cells [2], and was also reported to be reliable enough to be considered as a possible early diagnostic biomarker in cases of acute kidney injury [3].

Sox10 is expressed strongly in emerging neural crest cells and later in developing glial cells of the enteric nervous system (ENS) but not in neuronal cells. Thus, $Sox10^{+}$ cells can be used as one of the earliest markers of neural crest cell activity in the ENS. In Sox10-Venus/C57BL GFP transgenic mice (Sox10-Venus mice), intestinal neural crest-derived cells can be visualized with modified enhanced green fluorescent protein (Venus) without any histochemical staining according to a protocol described by Shibata et al. [6]. Knowing that there is a well-known relationship between glial cells and neurons and that Sox10 is expressed strongly in emerging neural crest cells and later in developing glial cells of the enteric nervous system, it seemed logical that visualizing glial cells easily would be advantageous and accordingly chose to use Sox10-Venus mice for this study.

A number of studies have assessed the ENS after intestinal I/R injury on the basis that motility and fluid transport in the intestines are controlled by the ENS [4,5]. Structural damage to enteric neurons and enteric glia associated with I/R injury has been reported [5] but more comprehensive research is lacking. To the best of our knowledge, the regulation of Sema3A expression and its pathophysiological role in intestinal I/R injury have not been studied thoroughly and knowledge about it is unclear. Here we assessed the ENS in Sox10-Venus mice by using stereoscopic microscopy and a fluorescence microscopy under high/low magnification to assess the expression and distribution of Sema3A after intestinal I/R injury.

Materials and methods

Animals

Sox10-Venus mice (18-21g) raised according to a protocol described by Shibata et al. were used to assess the ENS after intestinal I/R [6]. All animal procedures were reviewed and approved by the Institutional Review Board (IRB) of the Juntendo University School of Medicine Animal Care and Use Committee (Institutional Review Board No. 280082).

Surgical technique for ischemia/reperfusion injury

Anesthesia was induced and maintained with inhaled isoflurane. A midline laparotomy was used to exteriorize a loop of ileum and a 20-30mm long segment from the ileocecal valve that was supplied by single branches of the superior mesenteric artery and vein was marked. The branch of the superior mesenteric vein, marginal arteries and veins supplying the segment, and the wall of the bowel at the ends of the chosen intestinal segment were occluded with an atraumatic vascular clamp for 3 hours according to a technique reported by Thacker [5]. Both ends of the segment were marked with 4-0 nylon on the mesenteric side. During clamping, the abdomen was covered with sterile plastic wrap and a light was used to prevent hypothermia. Reperfusion was induced by removing the clamp. The loop of ileum was returned to the abdomen and repositioned and laparotomy incision closed with a running 3-0 nylon suture [5].

Specimen harvesting

Mice were sacrificed 0, 3, 12, 24, 48, and 96 hours after reperfusion by cervical dislocation and the I/R segment harvested. The number of mice sacrificed for each time group was (n=2) for the 3, 24, and 48 hour groups and (n=3) for the 0, 12, and 96 hour groups. Ileum proximal to the I/R segment was used as a control segment in each mouse. The middle portion of each harvested I/R segment (15–20 mm) was incised and used for stereoscopic and fluorescence microscopy.

Specimens were not washed before tissue fixation. The only preparation was to remove obvious feces from specimens before taking 5 random sections from each

specimen. We were concerned that uptake of antibodies by damaged tissue may create a false positive result so harvested specimens were handled carefully and all procedures were conducted in a uniform way under standard conditions. Thus, we do not believe tissue handling influenced our results.

Histochemistry

Harvested specimens were immersed in 30% sucrose, embedded in OCT compound (Sakura Finetek, USA) and sectioned 10μm thick. Hematoxylin and eosin (HE) staining was performed using conventional techniques. Nuclear counterstaining was performed with 4′,6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA). Sections were incubated with anti-Sema3A (Santa Cruz Biotechnology, Inc, 1:50, USA) and anti-protein gene product 9.5 (PGP9.5) (rabbit polyclonal, 1:500, Enzo Life Science, USA) for assessing nerve cells. Control staining was performed by omitting the primary antibody as a negative control.

Sema3A expression

The intensity and distribution of Sema3A in the muscular layers of harvested specimens were analysed blindly in five random sections from each specimen by using a predetermined standard for assessing the presence of Sema3A. Intensity for a specimen was the average of data that exceeded the predetermined standard collected from each of 5 sections assessed blindly, and distribution was the percentage of intensity results that exceeded the standard.

To quantify our data, we photographed five random sections from each specimen using the same LSM 780 laser-scanning microscope (ZEISS, Jena, Germany) under the same conditions (Master gain: 560 ChS2: 765, Lasers 560 560nm: 2.3%). The intensity and distribution of Sema3A in the muscular layers of harvested specimens were quantified by analyzing 5 random sections from each specimen blindly using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016).

In this study, Sema3A distribution was not assessed according to specific cell type. We only investigated the intensity and distribution of Sema3A in the muscle layers on the assumption that if villi were severely damaged by ischemia/reperfusion, then it would be difficult to examine the mucosa and even harder to evaluate Sema3A in villi.

Asassessment of the ENS

The number of ganglia in each specimen were counted under fluorescence microscopy without histochemical staining. Results were divided by the circumference of each specimen in millimeters for standardization. The number of Sox10-Venus^{+} cells and PGP9.5 positive cells (PGP9.5^{+} cells) in ganglia per high power field were also counted in each harvested ileal specimen.

On HE staining, changes to neurons appeared to be only slight and non-specific. We did try immunostaining for Hu protein, known to be an early marker of neuronal phenotypic differentiation in this study, but our results were inconsistent. We plan to conduct further research using our model with other neuronal markers such as Hu protein.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical evaluation of differences between control and intestinal I/R segments was by the Student's t test for unpaired comparisons. One-way analysis of variance (ANOVA) was followed by Tukey's post hoc test for multiple comparisons using SPSS (IBM Corp. Released 2008. IBM SPSS Statistics for Windows, Version 17.0. Armonk, NY: IBM Corp). Statistical significance was defined as $p < .05$.

Acknowledgement

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Results

Sema3A expression and Hematoxylin and eosin (HE) staining

The intensity and distribution of Sema3A in the muscular layer of longitudinal sections of ileum were significantly higher at 3 and 12 hours after reperfusion than in specimens harvested at other times ($p < .05$, $p < .05$, respectively) (Fig. 1: A-G). Injury to the mucosa observed under HE staining at 3 and 12 hours was also more serious than in specimens harvested at other times (Fig. 1: H-N). After 3 hours, both the intensity and distribution of Sema3A decreased gradually, reverting to original levels by 96 hours after reperfusion (Fig. 1). Fig. 2 summarizes the intensity and distribution of Sema3A observed in all specimens.

Sox10-Venus$^+$ cells under stereoscopic microscopy

Sox10 had faded in specimens harvested at 0, 3, and 12 hours after reperfusion (Fig. 3: A-G). Reversion to the dense distribution of Sox10 seen originally was gradual and complete in specimens harvested 96 hour after reperfusion.

Sox10-Venus$^+$ cells and PGP9.5$^+$ cells under fluorescence microscopy

The number of Sox10-Venus$^+$ cells was significantly decreased ($p < .05$) with no obvious ganglia 0 hours after reperfusion (Fig. 3: H-U). However, the number of ganglia visualized at 96 hours was significantly greater than in controls ($p < .05$), but the number of Sox10-Venus$^+$ cells per ganglion at 96 hours was significantly less ($p < .05$) than controls (Fig. 4). Although there were less PGP9.5$^+$ cells seen 24 and 48 hours after reperfusion, differences were not statistically significant ($p = .53$, $p = .58$ respectively) (Fig. 5)

Discussion

Sema3A is known to influence cell migration and significant up-regulation of Sema3A has been reported after reperfusion in acute kidney injury [3]. Specifically, Sema3A expression was increased 24 hours after reperfusion in kidney tissue and the quantity of Sema3A in urine was increased at 6 and 24 hours after reperfusion [3]. Although the mechanism of enhanced excretion of Sema3A following I/R injury is not

clear, an interaction between Sema3A, activated T cells and dendritic cells [1] was purported to be the etiologic mechanism [4]. In our study, increased expression of Sema3A was also recognized early after reperfusion (at 3 and 12 hours) followed by gradual down-regulation to original levels by 96 hours after reperfusion. HE staining also identified tissue damage at 3 and 12 hours that correlated with Sema3A changes consistent with acute kidney I/R injury findings (Fig. 1).

The progression of changes in the ENS following I/R injury can be visualized clearly in Sox10-Venus mice and to the best of our knowledge we are the first to monitor changes to the ENS in Sox10-Venus mice under stereoscopic microscopy without histochemical staining (Fig. 3: A-G). In other words, the significant fading in Sox10-Venus$^+$ cells at 0 and 3 hours, followed by gradual up-regulation after 24 hours that could be observed clearly under stereoscopic microscopy was even more obvious under fluorescence microscopy because of greater sensitivity under both high and low power magnification (Fig. 3: H-U). Thus, while the number of ganglia had increased significantly by 96 hours after reperfusion ($p < .05$), the number of Sox10-Venus$^+$ cells per ganglion at 96 hours was significantly less than in controls ($p < .05$) (Fig. 4) indicating that the ENS had still not recovered completely by 96 hours after reperfusion suggesting we were in fact observing one stage in the recovery process.

An interesting point in this study was there was no change in PGP9.5$^+$ cells observed in this study. As a marker of nerve cell regeneration we expected some response but the lack of reaction would suggest that recovery from I/R injury may only involve glial cells as reflected by changes observed in Sox10-Venus$^+$ cell expression/distribution as opposed to nerve cells, or that nerve cells may be more resistant to I/R injury than glial cells. Thacker et al [5] compared the number of neurons within ganglia containing distorted glial cells with the number of neurons within ganglia of normal appearance after the same amount of time had passed. They did not compare changes in the number of neurons over time as we did. They also reported nerve cell damage after intestinal I/R but we could not confirm such findings in this study although there was a trend for the number of neurons to decrease but it was not statistically significant. When we planned our study we thought that simple

comparisons would be difficult and that changes over time might hint at the presence of a correlation or a trend.

We believe there is a correlation between tissue damage as observed on HE staining and Sema3A expression and Sox10-Venus^{+} cell distribution following intestinal I/R injury because there were similar trends between Sema3A expression and ENS damage observed although they did not occur at exactly the same time; that is, the Sox10-Venus^{+} cell response was more sensitive with significant changes being observed at 0 hours after reperfusion while Sema3A took longer to respond.

We are the first to show there is increased expression of Sema3A following intestinal I/R injury by observing changes to the ENS in Sox10-Venus mice. Further research on the mechanisms involved in Sema3A expression, altered ENS and responses made by the body during recovery are warranted to clarify the exact role of Sema3A in the recovery response to intestinal I/R injury.

References

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Figure Legends

Fig. 1 Expression of semaphorin3A (Sema3A) (A-G: x63, double headed arrows indicate the muscular layer; Scale bars = 20μm) and Hematoxylin and eosin (HE) staining (H-N: x10, Scale bars = 100μm).

At 3 and 12 hours after reperfusion, differences in Sema3A expression were statistically significant. Histologic damage on HE staining was most severe at these times.

Fig. 2 Intensity and distribution of Semaphorin3A.

Semaphorin3A expression was significantly greater at 3 and 12 hours after reperfusion. The distribution of Semaphorin3A at 3 hours was significantly greater than in control segments. Mean ± SE. * $p < .05$.

Fig. 3 Assessment of Sox10-Venus^{+} cells under stereoscopic microscopy and fluorescence microscopy.

Stereoscopic microscopy: (A-G: x2, Scale bars = 500μm)

Fluorescence microscopy:

Low magnification: (H-N: x5, Scale bars = 200μm)

High magnification: (O-U: x63, Scale bars = 20μm, O-U: x2 digital magnified section, Scale bars = 10μm)

Merged view: Semaphorin 3A (red), DAPI (blue), and Sox10 (green).

A, H, and O: controls; B, I, and P: at 0 hours; C, J, and Q: at 3 hours; D, K, and R: at 12 hours; E, L, and S: at 24 hours; F, M and T: at 48 hours; G, N, and U: at 96 hours, respectively.

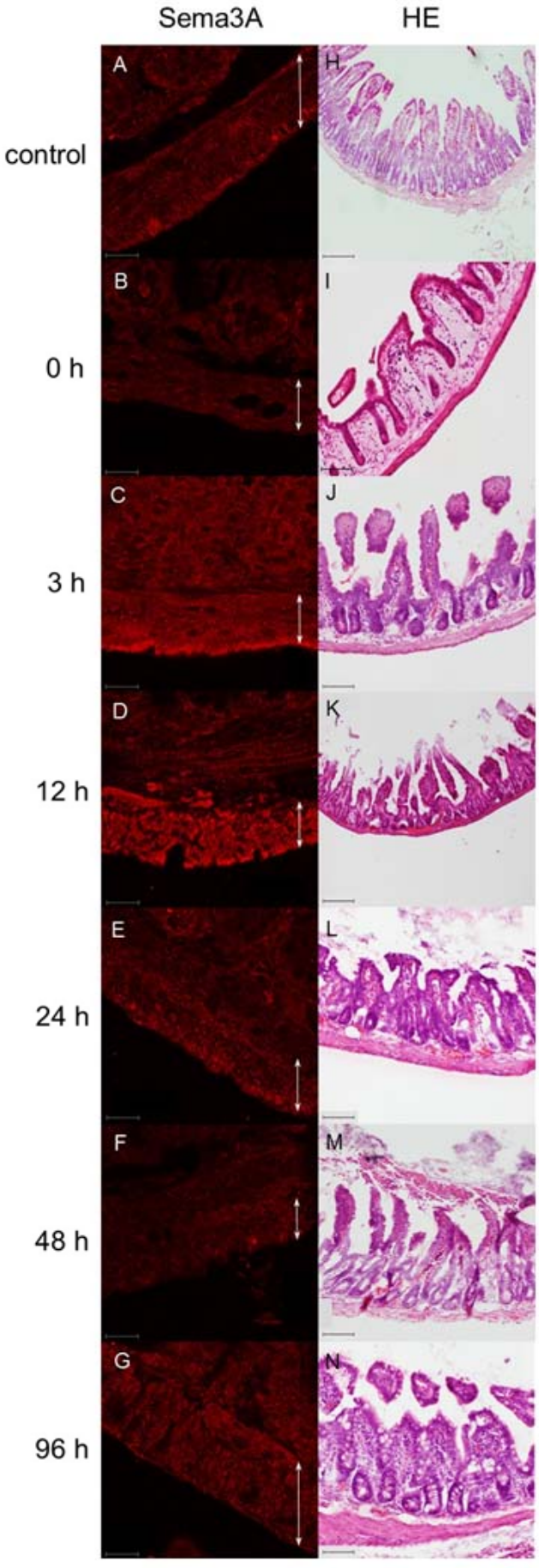
Sox10-Venus^{+} cells had faded significantly at 0 and 3 hours after reperfusion and reverted to original levels by 96 hours after reperfusion (G, N, and U).

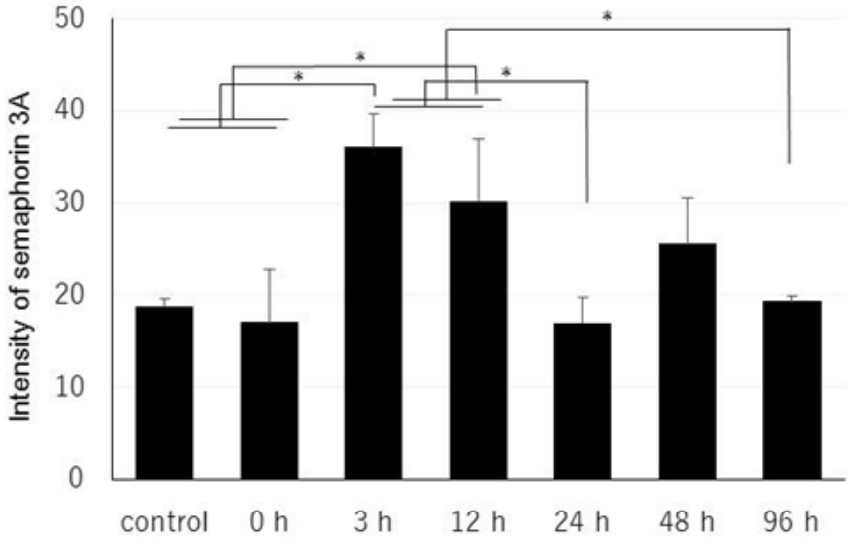
Fig. 4 Number of ganglia expressed per millimeter of circumference and the number of Sox10-Venus^{+} cells per ganglion.

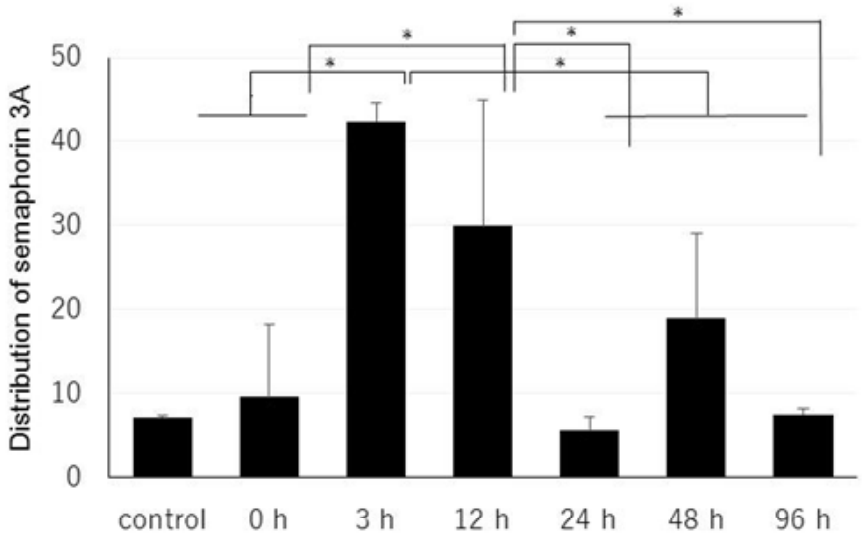
The number of ganglia in segments 96 hours after reperfusion was significantly higher than in control segments. Mean ± SE. * $p < .05$.

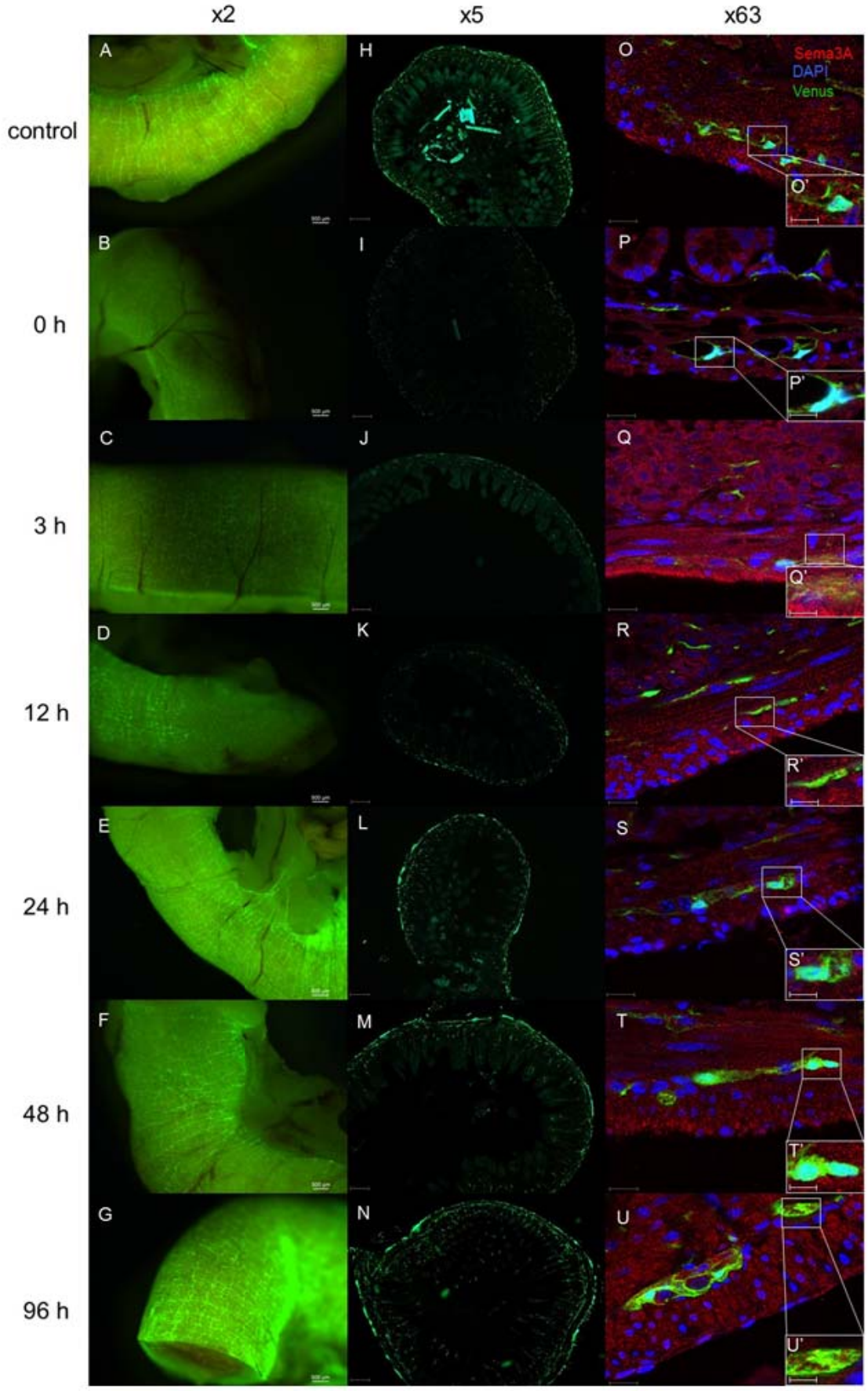
Fig. 5 Number of PGP9.5^{+} cells per ganglion.

There were less PGP9.5^{+} cells at 24 and 48 hours after reperfusion, but differences were not statistically significant. Mean ± SE.


Sema3A
HE
control
0 h
3 h
12 h
24 h
48 h
96 h
A
B
C
D
E
F
G
H
I
J
K
L
M
N


Intensity of semaphorin 3A
50
40
30
20
10
0
control
0 h
3 h
12 h
24 h
48 h
96 h
*
*
*
*
*


Distribution of semaphorin 3A
50
40
30
20
10
0
control
0 h
3 h
12 h
24 h
48 h
96 h
*
*
*
*
*


x2
x5
x63
control
0 h
3 h
12 h
24 h
48 h
96 h
A
B
C
D
E
F
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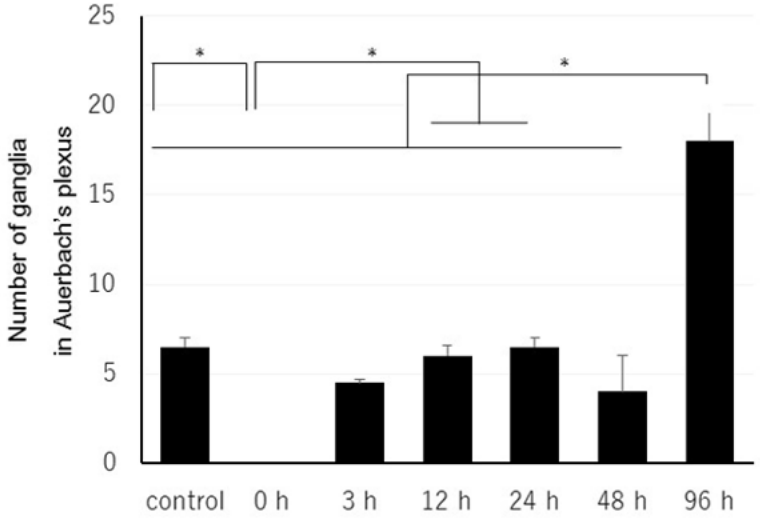
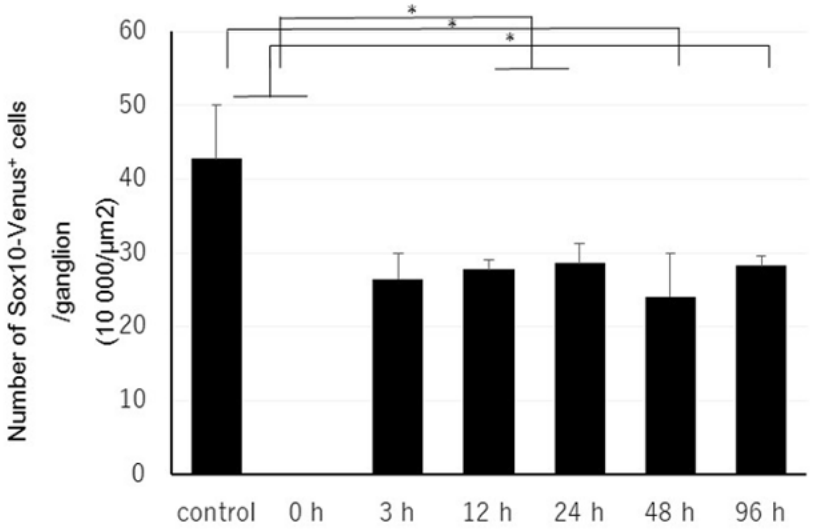
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
O'
P'
Q'
R'
S'
T'
U'
Sema3A
DAPI
Venus


Number of ganglia
in Auerbach's plexus
25
20
15
10
5
0
*
*
*
control
0 h
3 h
12 h
24 h
48 h
96 h


Number of Sox10-Venus+ cells
/ganglion
(10 000/μm2)
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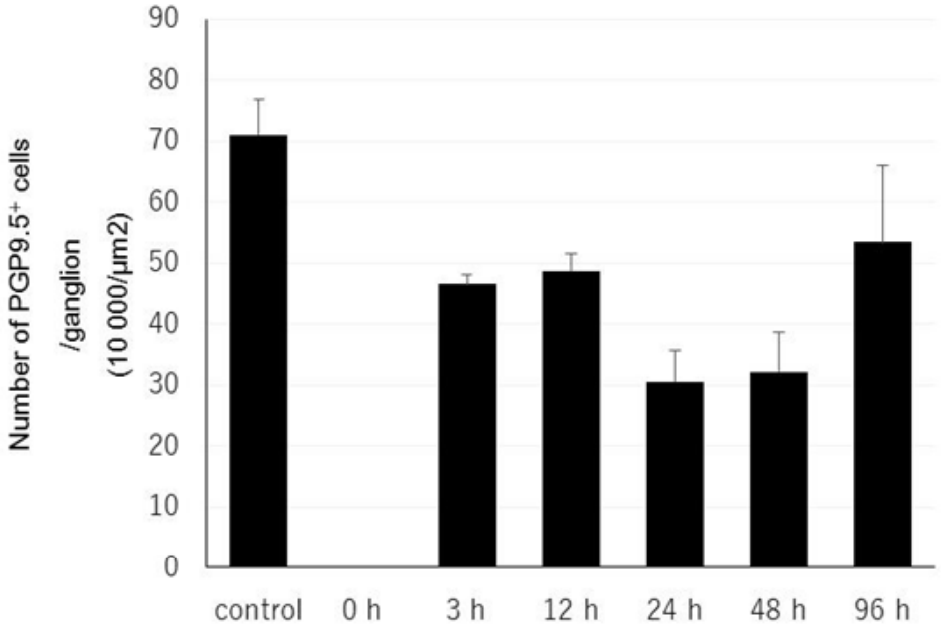
50
40
30
20
10
0
*
*
*
control
0 h
3 h
12 h
24 h
48 h
96 h


Number of PGP9.5+ cells
/ganglion
(10 000/μm2)
90
80
70
60
50
40
30
20
10
0
control
0 h
3 h
12 h
24 h
48 h
96 h