Evaluation of the neuroprotective effect of minocycline in a rabbit spinal cord ischemia model

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Conflict of Interest: There are no conflicts of interest to disclose.

Financial Support: Supported by Grant-in Aid for Scientific Research C2-22591715 in Japan

*Structured Abstract

Abstract

Objective: To investigate whether post-ischemic administration of minocycline attenuates hind-limb motor dysfunction and gray and white matter injury after spinal cord ischemia.

Design: A prospective, randomized laboratory investigation

Setting: Laboratory in university, single-institutional

Parcitipants: Male New Zealand White rabbits

Intervention: Spinal cord ischemia was induced by an occlusion of infrarenal aorta for 15 min. Group M1 (n=8) with a minocycline administration 1 hour after reperfusion; Group M3 (n=8) with a minocycline administration 3 hours after reperfusion; Group C (n=8) with a saline administration 1 hour after reperfusion or Sham group (n=4). Minocycline was intravenously administered at 10 mg/kg a total of 6 times at 12-hour intervals until 60 hours after the initial administration.

Measurement and Main results: Hind limb motor function was assessed using Tarlov score. For histological assessments, gray and white matter injury was evaluated 72 hours after reperfusion using the number of normal neurons and percentage areas of vacuolations, respectively. The motor function 72 hours after reperfusion was significantly better in the

Group M1 than in the Group C. The number of neurons in the anterior horn was significantly higher in the Group M1 than in the Group M3 or C, but did not significantly differ between Groups M3 and C. No significant difference was noted in the percentage areas of vacuolations among the ischemia groups.

Conclusions: Minocycline administration beginning at 1hour after reperfusion improved hind-limb motor dysfunction and attenuated gray matter injury in a rabbit spinal cord ischemia model.

Key words: Minocycline, ischemic spinal cord injury, reperfusion, neuroprotective effect, rabbit

Introduction

An important complication of thoracic or thoracoabdominal aortic aneurysm is lower limb paralysis due to spinal cord ischemia (SCI)（1, 2）. Hypothermia therapy, spinal cord drainage, and various drug therapies to protect the spinal cord from ischemia have been reported (3, 4), but their effects are limited. Therefore, further efforts to clarify the mechanisms of nerve injury and develop neuroprotective drugs are being made.

Minocycline is a semi-synthetic tetracycline antibiotic that passes the blood-brain barrier and enters the central nervous system, and is noted for its biological other than antibiotic actions (5-8). It has been reported to reduce the size of ischemic foci using brain ischemia models (9-12). On the basis of these reports, an open-label trial was conducted in acute stroke patients, showing a more favorable outcome compared with the placebo group (13). In spinal cord injury models, minocycline has been shown to alleviate the impairment of spinal cord tissue and improve motor functions in contusion and compression models (14-17).

Our previous study indicated that intraperitoneal administration of minocycline before

ischemia improved the hind limb motor function and alleviated impairment of the gray and white matter in a rat SCI model (18). However, there are no data available on minocycline-mediated neuroprotection when administered after reperfusion in a model of SCI. The present study was conducted to examine whether minocycline administration after reperfusion also has a neuroprotective effect in a rabbit model of SCI, and evaluated the therapeutic window period of this effect.

Methods

This study was approved by the Animal Experiment Committee of Nara Medical University (Kashihara, Nara, Japan). Twenty-eight New Zealand White rabbits weighing 2.7-3.0 kg were used. These rabbits were housed under 12-hour light-dark cycles with free access to food and water.

Animal allocation

The rabbits were randomly allocated to one of the following 4 groups: Control (C) group (n=8), minocycline administration after 1 hour (M-1) group (n=8), minocycline administration after 3 hours (M-3) group (n=8), or sham group (n=4). In the M-1 and C groups, minocycline (Nichi-iko, Toyama, Japan) or normal saline was administered intravenously every 12 hours from 1 hour after reperfusion for 60 hours (a total of 6 times). In the M-3 group, minocycline was also administered every 12 hours for 60 hours, but the administration was started 3 hours after reperfusion. The dose of minocycline was 10 mg/kg (in 3 ml). The sham group was administered the same amount of normal saline employing the same schedule as in the C group without aortic occlusion.

Spinal cord ischemia

The rabbits were anesthetized in a plastic box using oxygen and 5% isoflurane. A catheter was inserted into the auricular vein, and Ringer's solution was administered at 10 ml/kg/h. The auricular artery was cannulated, and the proximal arterial pressure was measured. After the intravenous administration of fentanyl at 50 μg/kg, endotracheal intubation was carried out, and artificial ventilation (Harvard Respirator 510: Summit Medical, MA) was performed using 2-3.5% isoflurane, 30% oxygen, and air by adjusting the end-tidal carbon dioxide pressure to 35-40 mmHg. The rectal temperature was monitored continuously, and it was adjusted to 38-39°C using an electric mat. For measurement of the distal arterial pressure and blood sampling (blood gas and blood sugar analyses), the right femoral artery was exposed, and an SP-55 catheter was inserted. A 50-mm flank incision at the left costal level was made after infiltration of 1% lidocaine, and the aorta was exposed from the retroperitoneal cavity at the left renal artery level. A silicone thread 1.5 mm wide was carefully placed around the aorta immediately distal to the left renal artery, and both ends of the thread were passed through an occluder tube. Prior to the induction of ischemia, 600 U of heparin was administered. The

aorta was occluded by tightening the thread using the tube, and occlusion was confirmed by monitoring the distal pressure. After 15 minutes, aortic occlusion was released. After reperfusion, all catheters were removed, and the wound was closed. Blood was sampled 10 minutes before aortic occlusion and 10 minutes after reperfusion. The blood pressure and body temperature were measured 10 minutes before aortic occlusion, during the ischemia (7.5 minutes after aortic occlusion), and 10 minutes after reperfusion.

Neurological evaluation

The hind limbs of the rabbits were neurologically evaluated 24, 48 and 72 hours after reperfusion by a blinded observer. Assessment was made using Tarlov scoring (19), which consists of a five-point grading scale : 0 = paraplegic with no lower extremity function; 1 = poor lower extremity function, weak antigravity movement only; 2 = some lower extremity motor function with good antigravity strength but inability to draw leg under the body or hop; 3 = ability to draw legs under the body and hop but not normally; 4 = normal motor function.

Histological evaluation

Following the neurological evaluation after 72 hours, 50 mg/kg of thiopental was administered intraperitoneally, and the animals were anesthetized deeply using 5% isoflurane. After 1,000 ml of cold heparinized physiologic saline was infused transcardially, 500 ml of 4% paraformaldehyde in 0.1M PBS was administered. The lumbar spinal cord was removed, immersed in paraformaldehyde for 2days, the L5 part was cut off, embedded in paraffin, and sliced at a thickness of 3 μm for hematoxylin and eosin staining.

Gray matter injury was evaluated in terms of the number of normal neurons remaining in the left anterior horn of the spinal cord. An observer not informed of the randomization results counted normal neurons in an area of the left hemisphere anterior to the central canal of the spinal cord under light microscopy (×200). White matter injury was evaluated by examining the ventral, ventrolateral, and lateral areas and calculating the percentage areas of vacuolation, comprising the percentage of a 0.04-mm^2 area occupied by vacuoles. Each area was divided into 144 sub-areas using grid lines, a sub-area was regarded as a vacuolated area if 75% or more of it was occupied by vacuoles, and the percentage of vacuolated sub-areas in the 144 sub-areas was calculated. The total percentage of areas of vacuolation was calculated as a

mean of the 3 areas on each side.

Statistical analysis

The physiologic variables, number of normal neurons, and percentage areas of vacuolation were analyzed employing one-factor analysis of variance, and Fisher's PLSD was used as a post-hoc test. The motor function of the hind limbs was examined with the Kruskal-Wallis test, and the Mann-Whitney U-test was used as a post-hoc test. P value less than 0.05 was considered significant.

Results

Physiological variables are shown in table 1. There were no significant differences in the body weight or rectal temperature among the groups. Blood pressure values were similar among the groups before aortic occlusion, however, were significantly higher in the sham group than the ischemic groups during ischemia. Blood gas data are shown in table 2. No significant difference was noted in any of the blood test items including the pH, $PaCO_2$, PaO_2, hematocrit, and blood glucose level among the groups.

Tarlov Scores 24, 48 and 72 hours after reperfusion were shown in table 3. Figure 1 shows the individual neurologic scores 72 hours after reperfusion. Tarlov Scores in the sham group were significantly higher compared with those in the 3 groups exposed to ischemia. Tarlov Scores 72 hours after reperfusion were significantly higher in the M-1 group compared with those in the control group. No significant differences in Tarlov Score were noted between the C and M-3 groups.

Histograms of the numbers of neurons per animal and representative photomicrographs of

hematoxyline and eosin-stained section were shown in figure 1 and 2, respectively. The numbers of normal neurons were shown in figure 3. The number of normal neurons was significantly higher in the sham group than those in the other 3 groups. The number of normal neurons in the M-1 group was significantly higher than those in the control group. There were no significant differences in the number of normal neurons between the C and M-3 groups. Figure 4 shows the total percentage areas of vacuolation in each group . Percentage areas of vacuolation in the C group were significantly higher than in the sham group. While no significant differences were noted among the 3 groups exposed to ischemia, the percentage areas of vacuolation were lower in the M-1 group than in the C group.

Discussion

The results in the present study showed that intravenous administration of minocycline for 3 days at an interval of 12 hours, beginning at 1 hour, but not 3 hours, after reperfusion, significantly improved the hind limb motor function and significantly increased the number of normal neurons in the anterior horn 72 hours after reperfusion in rabbits subjected to spinal cord ischemia for 15 min, while the percentage areas of vacuolation were not affected by the administration of minocycline. These results suggest that minocycline administration after reperfusion can be neuroprotective, but early initiation of administration would be necessary for the treatment to be effective.

Although there has been no data available regarding the post-ischemic administration of minocycline in a model of spinal cord ischemia, neuroprotective effects of minocycline have been shown in models of cerebral ischemia (9-11, 20). Yrjänheikki et al. (9) reported that intraperitoneal administration of minocycline, beginning at 12 hours before ischemia or 30 minutes after reperfusion increased the number of remaining CA1 pyramidal neurons in a rat model of global cerebral ischemia. They also reported that monocycline administration,

beginning at 12 hours before ischemia or 4 hours after reperfusion significantly decreased the infarct volume after middle cerebral artery occlusion in rats (10).

Regarding the effects of minocycline on SCI, our previous report (18) in a rat aortic occlusion model is the first to our knowledge. We administered minocycline intraperitoneally at 45 mg/kg at an interval of 12 hours before ischemia, and reported the alleviation of impairment of the hind limb motor function and white and gray matter injury after 72 hours. The results in the present study indicated a neuroprotective effect of minocycline, which was administered 1 hrs, but not 3 hrs, after reperfusion in a rabbit model of SCI.

In the present study, a rabbit model of SCI was used due to the following reasons. First, in a rat SCI model, it has been reported that motor function gradually improved without no delayed deterioration, while hind-limb motor function deteriorated after SCI over 2 weeks in a rabbit model (21). Delayed development of paraplegia is well known in human, so that the time course after SCI in human may resemble to that in rabbit. Second, peritoneal administration is not appropriate for further clinical application and for accurate assessment of

therapeutic window due to variation in the absorption rate (5, 8). Repetitive intravenous administration could be performed if a rabbit model was used.

The dosage of minocycline in the present study was determined in the following reasons. The blood level of minocycline is considered to be similar after intravenous administration at 20 mg/kg and intraperitoneal administration at 90 mg/kg in rats (8). Xu L et al. (11) reported that the blood minocycline level on intravenous administration at 3 mg/kg in rats was equal to that at 200 mg in humans, which is a standard dose for humans. In the present study, we set the dose for intravenous administration at 10 mg/kg every 12 hours on the basis of the dose used in rabbits in expectation of an antibiotic effect (6 mg/kg, i.v., every 8 hours) (22). As a result, intravenous administration of minocycline at this dosage for 3 days at an interval of 12 hours, beginning at 1 hour, but not 3 hours, after reperfusion, significantly improved neurological outcome and alleviated gray matter injury 72 hours after reperfusion.

Although exact mechanisms of minocycline-mediated neuroprotective effects in this study are unknown, possible mechanisms may include anti-inflammatory actions due to the suppression

of microglia activation and subsequently decreasing the release of cytokines and chemokines (10,15,23-25), anti-apoptotic actions (26,27) due to inhibition of cytochrome C release(17) and caspase expression (28,29), inhibition of proNGF (Nerve growth factor) production (30), prevention of toxicity of glutamic acid (10), suppression of NO synthetase (22), and inhibitions of p38MAPK activation in microglia (22, 25, 30). However, since we did not perform the study to clarify the mechanisms of minocycline-mediated neuroprotection, further studies would be required.

There are several limitations in this study. First, concerning the dose, minocycline might also have been effective in the M-3 group if it had been administered at a higher dose or different regime for administration. Second, concerning the severity of ischemia, the results may have changed depending on the severity of SCI. However, the data of our previous studies indicated that 13-15 minutes time of ischemia is appropriate to evaluate the effect of neuroprotection. Aortic clamping for more than 20 minutes is too severe to destroy the spinal cord tissue. Third, the group in which minocycline was administered before ischemia, was not included in this study. So it is unclear whether administration of minocycline before ischemia

is more effective than that after reperfusion. Fourth, regarding the white matter injury, the evaluation at more than 4 days but not 72 hours after reperfusion might be appropriate. Kurita et al. (21) reported that white matter injury was unclear 24 hours after reperfusion but became clear after 4 days in a rabbit SCI model. Fifth, demonstration of neuroprotection in animal species does not necessarily translate to humans. Finally, concerning the long-term outcome, evaluation of the motor function and histological changes after a long-term interval after reperfusion would be necessary.

In summary, the effects of the intravenous administration of minocycline on white and gray matter injury and motor function were evaluated in a rabbit 15-min SCI model. As a result, an improvement in the motor function and alleviation of gray matter injury were observed after 72 hours when the drug was administered 1 hour after ischemia, but not when it was administered after 3 hours. Since minocycline is widely used clinically as an antibiotic, sufficient evaluation of its use as a drug for the prevention of paraplegia associated with surgery for thoracoabdominal aortic aneurysm is justified. While further evaluation would be necessary concerning its safe and effective use, its administration early after the onset of

ischemia may be one of alternative strategies to prevent further development of ischemic spinal cord injury.

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Figure legends

Figure 1

Histograms of the Tarlov score after 72 hours and the numbers of neurons per animal in the sham, control (C), minocycline administration after 1 hour (M-1), and minocycline administration after 3 hours (M-3) groups.

Figure 2

Representative photomicrographs of hematoxylin and eosin-stained section in the anterior horn of the spinal cord in the sham (a), the control (b), the M-1 (c), and the M-3 (d) groups. Normal neurons were better preserved in the M-1 group compare with those in the control and the M-3 groups.

Figure 3

The numbers of normal neurons in the sham, control (C), minocycline administration after 1 hour (M-1), and minocycline administration after 3 hours (M-3) groups. Of the 3

groups exposed to ischemia, the number of remaining neurons was higher in the M-1 group than in the other 2 groups ($p<0.05$).

Figure 4

The total percentage areas of vacuolation in the sham, control (C), minocycline administration after 1 hour (M-1), and minocycline administration after 3 hours (M-3) groups. It was significantly higher only in the C group than in the sham group ($P<0.05$). No significant difference was noted among the 3 groups exposed to ischemia.

Table 1

Table 1. Physiological variables

	Sham (n=4)	C (n=8)	M-1 (n=8)	M-3 (n=8)
Weight (kg)	2.9±0.1	2.8±0.1	2.8±0.1	2.9±0.1
Mean arterial pressure (mmHg)				
Before	67±7	63±5	60±11	65±7
During	66±10	12±2 a	12±1 a	12±1 a
After	ND	54±10	52±7	57±4
Rectal temperature (℃)				
Before	38.7±0.2	38.6±0.4	38.5±0.3	38.6±0.3
During	38.3±0.3	38.7±0.3	38.5±0.2	38.7±0.3
After	38.4±0.4	38.6±0.3	38.7±0.3	38.5±0.2

Data are expressed as mean±SD. ND; no data.

Mean arterial pressure and rectal temperature were measured 10 minutes before aortic occlusion (before), during ischemia (7.5 minutes after aortic occlusion) (during) and 10 minutes after reperfusion (after).

a; $P<0.05$ vs. Sham.

Table 2

Table 2. Blood gas data

	Sham (n=4)	C (n=8)	M-1 (n=8)	M-3 (n=8)
pH				
Before	7.49±0.04	7.46±0.05	7.50±0.07	7.4±0.05
After	7.46±0.01	7.44±0.06	7.45±0.05	7.4±0.04
PaCO2 (mmHg)				
Before	37±4	38±3	36±3	37±4
After	30±3	36±6	35±4	35±3
PaO2 (mmHg)				
Before	160±44	159±31	154±30	134±30
After	195±16	171±33	164±28	152±23
Hematocrit (%)				
Before	36±4	34±2	35±3	37±3
After	33±3	33±2	33±2	35±3
Glucose (mg/dl)				
Before	116±22	123±18	137±28	138±23
After	151±19	160±45	139±44	142±32

Data are expressed as mean±SD. Blood gas data. Blood was sampled 10 minutes before aortic occlusion (before) and 10 minutes after reperfusion (after). There were no significant differences among the groups. PaCO2 and PaO2; arterial patial pressure of carbon dioxide and oxygen, respectively.

Table 3

Table 3. Neurological outcome

	Tarlov score			
	Sham (n=4)	C (n=8)	M-1 (n=8)	M-3 (n=8)
24 hrs	4 (4-4)	2.5 a (2-3)	3 a (3-3.25)	2 a (2-2)
48hrs	4 (4-4)	2 a (2-2)	3 a (2-3)	2 a (2-2)
72hrs	4 (4-4)	2 a (2-2)	3 a,b (2-3)	2 a (2-2)

Data are expressed as median (25^{th}-75^{th}). a; $P<0.05$ vs. Sham, b; $P<0.05$ vs. C.

Tarlov score: 0 = paraplegic with no lower extremity function; 1 = poor lower extremity function, weak antigravity movement only; 2 = some lower extremity motor function with good antigravity strength but inability to draw leg under the body or hop; 3 = ability to draw legs under the body and hop but not normally; 4 = normal motor function.

Figure 1

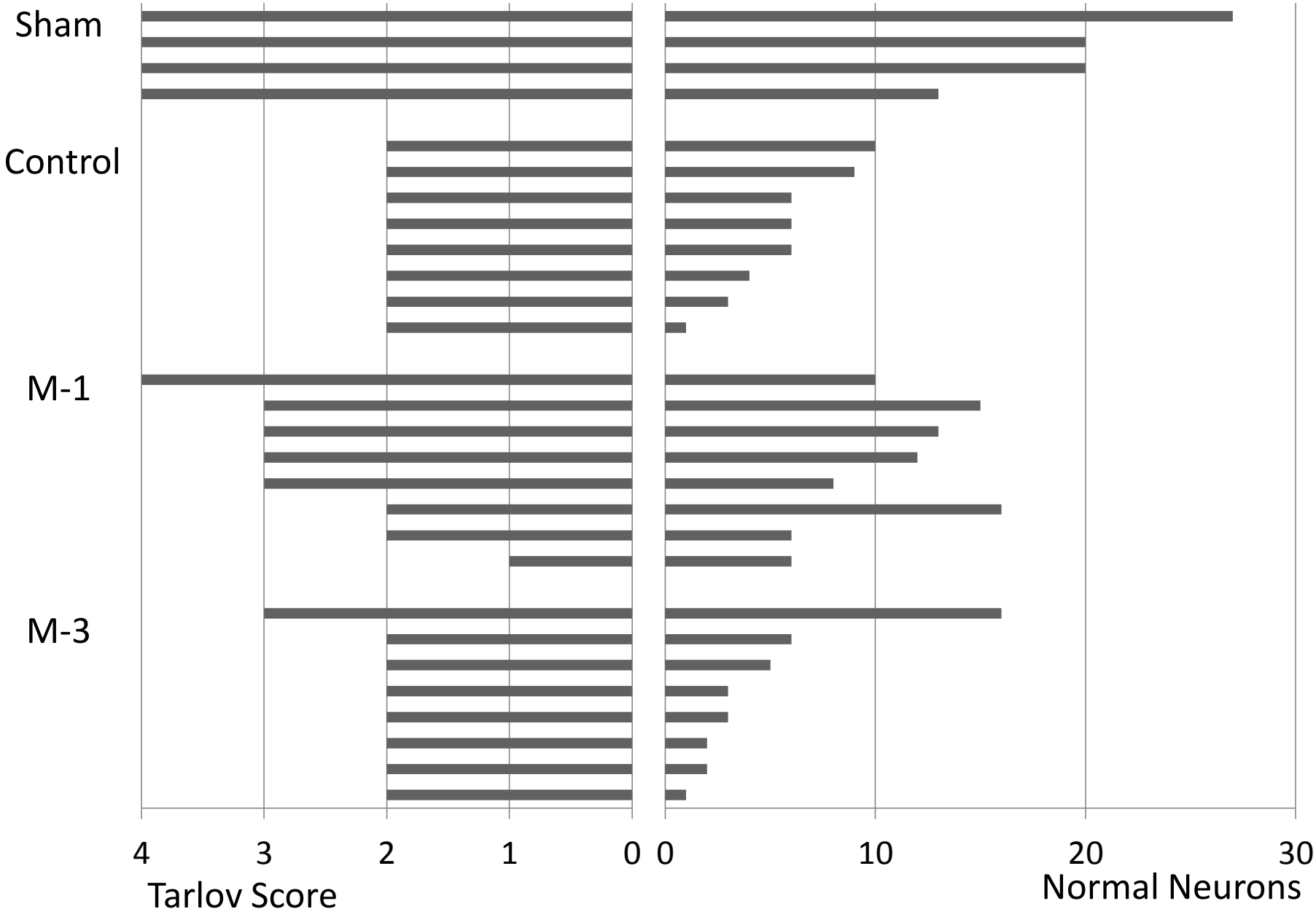

Sham
Control
M-1
M-3
4
3
2
1
0
Tarlov Score
0
10
20
30
Normal Neurons

Figure 2

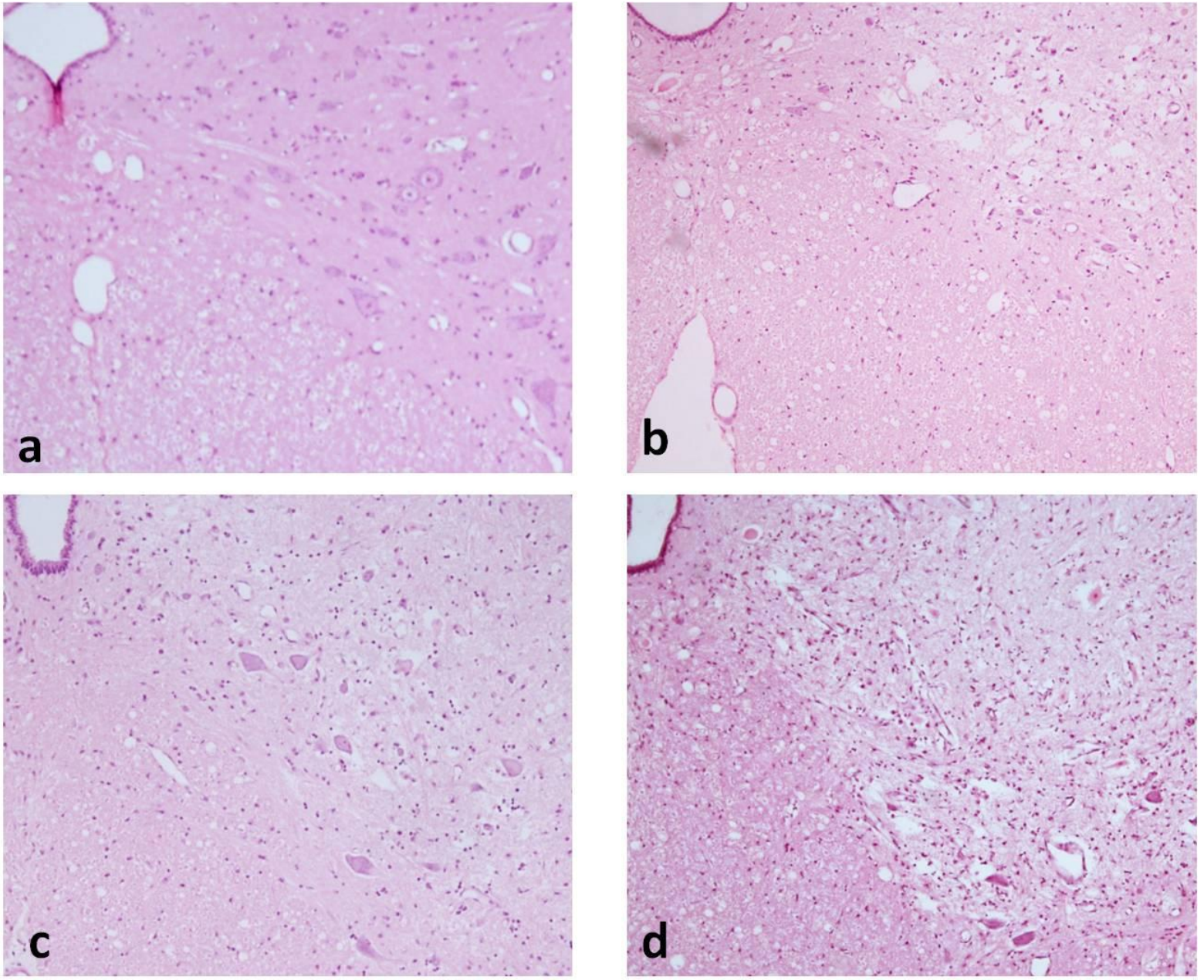


Figure 3

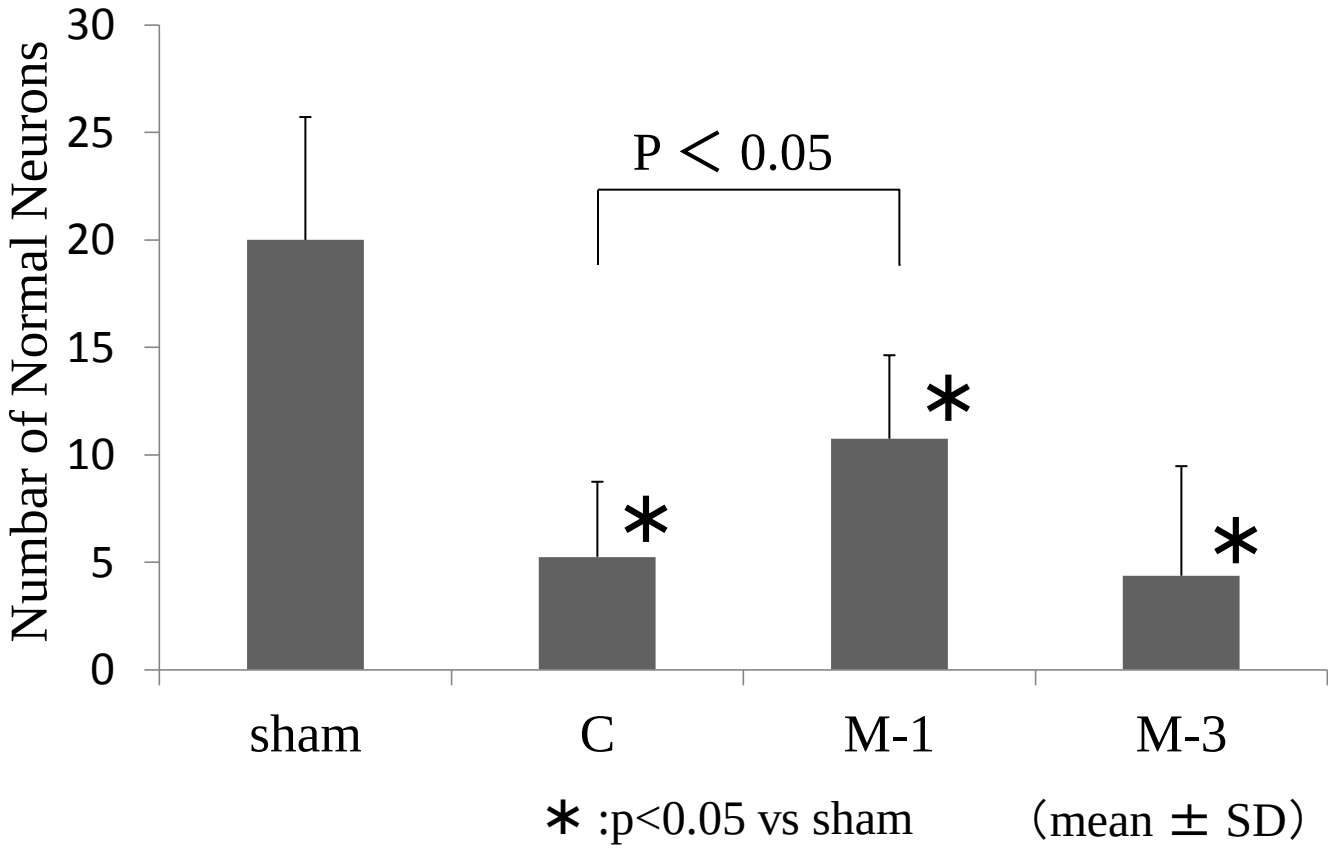
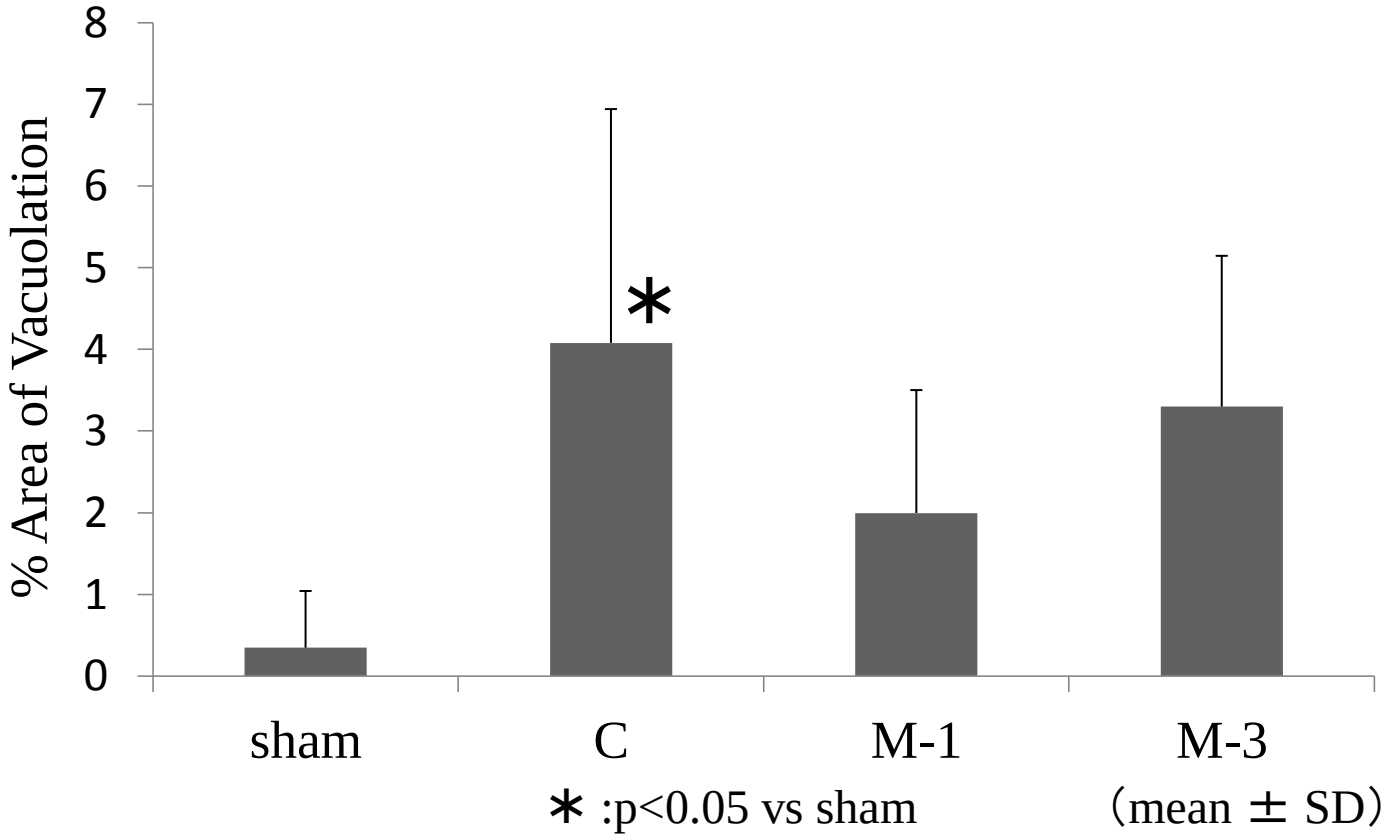

Numbar of Normal Neurons
30
25
20
15
10
5
0
P ＜ 0.05
＊
＊
＊
sham
C
M-1
M-3
＊ :p<0.05 vs sham
（mean ± SD）

Figure 4


% Area of Vacuolation
8
7
6
5
4
3
2
1
0
＊
sham
C
M-1
M-3
＊ :p<0.05 vs sham
（mean ± SD）