treatment. Subsequently, samples were embedded in paraffin, serial transverse sections were cut at 7 μm, and sections were pretreated with 0.3% H_2O_2 in PBS, rinsed in 0.1% Triton X-100 in PBS (T-PBS) and preincubated in 3% normal goat serum in T-PBS. Next, sections were incubated overnight at 4°C with mouse monoclonal antibody against ChAT (Chemicon, mouse anti-ChAT, Temecula, CA, USA) at a dilution of 1:100, followed by incubation with HRP-labeled anti-mouse polymer (Dako Cytomation, Carpinteria, CA, USA). Treated sections were visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution and counterstained with Hematoxylin.

2.4. Neurofunctional analysis

Motor nerve conduction velocity (MNCV) was calculated to assess the neurological functional recovery of RLN after crush injury. Animals were anesthetized and the left recurrent laryngeal nerve was exposed inferior to the left lobe of the thyroid gland, as described above. The strap muscles were sectioned to expose the larynx, and laryngeal fissures were made. The left thyroarytenoid (TA) muscle was pierced through the fissure with a needle concentric electrode for recording. To stimulate the left RLN, two bipolar hook electrodes were placed to hook the dissected left RLN. One was placed inferior to the left lobe of the thyroid as a distal stimulator and the other was placed 16 mm proximal to the distal electrode as a proximal stimulator. The nerve was maximally stimulated and

compound muscle action potential in TA muscle was recorded using a Power Lab computer-assisted electromyography machine (AD Instruments, Colorado Springs, CO, USA). Maximal stimulation was achieved by increasing the current output until no further changes in amplitude of the compound action potential occurred. A 0.01-millisecond current impulse was delivered. Maximum MNCV was calculated based on derived latency and distance between the two stimulating points (16 mm).

At the time of laryngeal fissure creation, recovery of vocal fold movement was also assessed. Recovery was only considered present when equal vocal fold movement on the denervated side was observed when compared to the vocal fold on the contralateral non-denervated side. Limited recovery was considered to be the absence of recovery.

2.5. Statistical analysis

Data are expressed as means ± S.D. Statistical comparison of motoneuron loss and MNCV were performed by Mann-Whitney U test. Recovery of vocal fold movement was statistically compared by χ^2-test for independence. The level of significance was set at $p<0.05$.

3. Results

3.1. Neuroprotective effects of T-588 administration

The left vagal nerves of adult rats were avulsed and removed at the level of the jugular foramen.

Animals were freely administrated water containing 0.05% T-588 solution after surgery. Four weeks after surgery, the number of surviving motoneurons in the nucleus ambiguus was counted using Nissl staining in order to evaluate the neuroprotective effects of T-588 (T-588, n=6; control, n=5). There was marked atrophy and loss of motoneurons in the nucleus ambiguus of the lesion side (Fig.1). The number of motoneurons decreased and reached 57.9±4.8% when compared with the contralateral side in the control group. Oral administration of T-588 successfully prevented the motoneuron loss, i.e., the number of residual motoneurons in the ipsilateral nucleus ambiguus (69.0±3.5%) was significantly higher in the treatment group when compared to controls (P=0.0062). (Fig. 2). ChAT immunoreactivity is known to rapidly decrease in the motoneurons after nerve injury.[15-19] Although marked decreases in immunoreactivity in the nucleus ambiguous was observed in the control group, improved ChAT immunoreactivity was observed in the treatment group at 4 weeks after vagal nerve avulsion (Fig. 3).

3.2. Neurofunctional recovery after T-588 administration

Effects of T-588 administration on neurofunctional recovery were examined at 4 weeks after RLN crush. Shorter latency, together with shorter time lag of latency was observed in the treatment group when compared to controls (Fig. 4). Mean (±S.D.) MNCV in the treatment group (32.07 ± 16 m/s) was significantly higher than in the control group (20.47 ± 5.02 m/s, P=0.015) (Fig. 5).

3.3. Recovery of vocal fold movement

The number of rats displaying obvious recovery of ipsilateral vocal fold movement was 9/12 in the treatment group and 2/9 in the control group. Statistically better recovery was observed in the treatment group when compared to controls (P=0.016) (Table. 1).

4. Discussion

T-588, a synthetic derivative of acetylcholine[20], has been developed as a candidate neuroprotective agent against neurodegenerative diseases. Clinical trials using T-588 to treat dementia associated with Alzheimer's disease are currently underway.[10] T-588 is efficiency transported into the central nervous system (CNS)[11], and it has been reported to delay the progression of Alzheimer's disease in wobbler mouse[11] and to exert neuroprotective effects against ischemia/reperfusion-induced brain damage in vivo[21]. Oral administration of T-588 improves the survival of injured motoneurons and supports their neuronal function after facial nerve avulsion.[11] In vitro, this compound enhances neurite outgrowth and ChAT activity in primary explant cultures of the ventral spinal cord[22] and activates the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) pathway in cultured rat newborn astrocytes, inhibiting astrocyte apoptosis induced by Ca^{2+}stress.[23] These data suggest that T-588 exerts neuroprotective effects in damaged motoneurons.

In this study, the vagal nerve avulsion model was utilized to assess the neuroprotective effects of T-588. Marked motoneuron loss was observed in the ipsilateral nucleus ambiguus after surgery, as reported previously.[7, 8] Similar findings have been reported in the facial nucleus after facial nerve avulsion and in the ventral horn after spinal root avulsion.[18, 19] Treatment with T-588 significantly prevented the loss of vagal motoneurons when compared to controls. The presence of ChAT is associated with the viability of motoneurons and ChAT immunoreactivity is known to decrease rapidly at 1 week after facial nerve or spinal nerve avulsion. Thereafter, ChAT immunoreactivity gradually decreases for 7weeks.[15-19] It has been reported that oral administration of T-588 after facial nerve avulsion improved ChAT immunoreactivity in adult rats.[12] In the present study, the decrease in ChAT immunoreactivity was attenuated by the T-588 treatment in the nucleus ambiguus. These findings are indicative of the neuroprotective effects of T-588 on vagal nerve motoneurons after severe vagal nerve injury.

Detrition injury to vagal/recurrent laryngeal nerves can be caused by surgery utilizing a cervical or mediastinal approach. Nerve crush consistently induces Sunderland second-degree injury (axonotmesis), yielding Wallerian degeneration of the nerve distal to the injury site.[24, 25] Bridge *et al.* demonstrated that the functional and histological responses to crush are identical in the various methods to deliver crush injury to the rat sciatic nerve.[24, 25] MNCV for the injured nerve is a commonly used physiological measure to evaluate functional recovery of peripheral nerves after

injury.[24-26] The present study assessed neurofunctional recovery at 4 weeks after crush injury to RLN. Treatment with T-588 resulted in significant improvement in MNCV and vocal fold movement when compared to control animals. It has been reported that it takes 8 weeks for physiological and histological recovery of peripheral nerves after crush injury.[24-26] Reducing the RLN functional recovery period with T-588 administration may ameliorate speech/swallowing problems more quickly and prevent atrophy of the internal laryngeal muscles.

5. Conclusion

We demonstrated that oral administration of T-588 prevents motor neuron loss in the nucleus ambiguus and supports neurofunctional recovery after vagal/recurrent laryngeal nerve injury. Oral administration of T-588 is thus a promising therapeutic approach for various peripheral injuries.

6.References

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7. Figure legends

Fig. 1.

Nucleus ambiguous after 4 weeks of T-588 administration.

Nucleus ambiguus on the contralateral side (a), ipsilateral side of the control group (b), and ipsilateral side of the T-588 treatment group (c) are shown. Clear inhibition of neuronal death was observed in the T-588 treatment group. Scale bar = 50 μm.

Fig. 2.

Relative percentage of surviving motoneurons after 4 weeks of T-588 administration.

Relative percentage of surviving motoneurons compared to contralateral side after 4 weeks of T-588 administration. Data are presented as means ± SD. Statistical comparison was performed by Mann-Whitney U test (*$p<0.05$).

Fig. 3.

ChAT immunohistochemistry of nucleus ambiguus.

Nucleus ambiguous of the contralateral side (a), ipsilateral side of the control group (b), and ipsilateral side of the T-588 treatment group (c) are shown. ChAT immunoreactivity was preserved in the T-588 treatment group. Scale bar = 50 μm.

Fig. 4.

Electromyography of thyroarytenoid muscle.

Representative EMG stimulating left RLN. RLN was stimulated at both the proximal (dotted line) and distal (continuous line) sides of the crushed nerve. The origin points of the first waves (arrows) in the control group (a) and T-588 treatment group (b) are shown. The action potential in the T-588 group rat is more obvious than in controls. Moreover, the latency and the interval of the 2 origin points of the waves in the T-588 group were lower than those in control rats.

Fig. 5.

MNCV at 4 weeks after nerve crush injury.

MNCV was significantly higher in the T-588-treated group than in controls, thus demonstrating the strong protective and regenerative effects of T-588 against motor nerve injury. Data are presented as means $\pm$SD. Statistical comparison was performed by Mann-Whitney U test ($*p<0.05$).

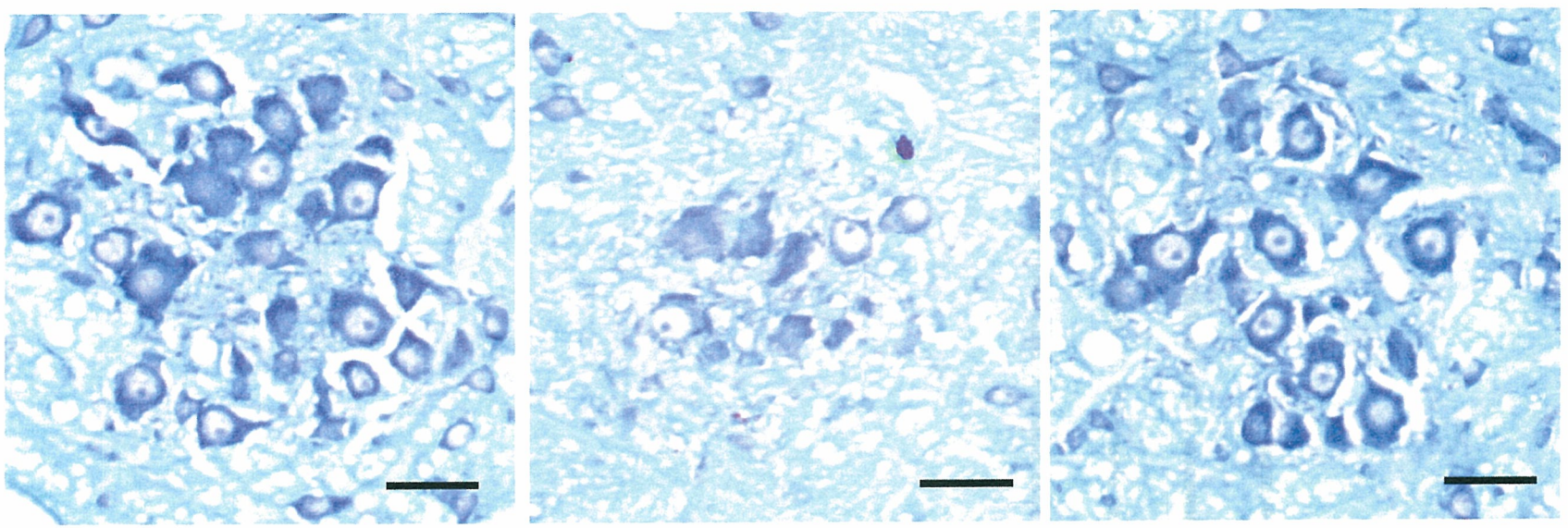
8.Table

Table 1.

Recovery of vocal fold movement.

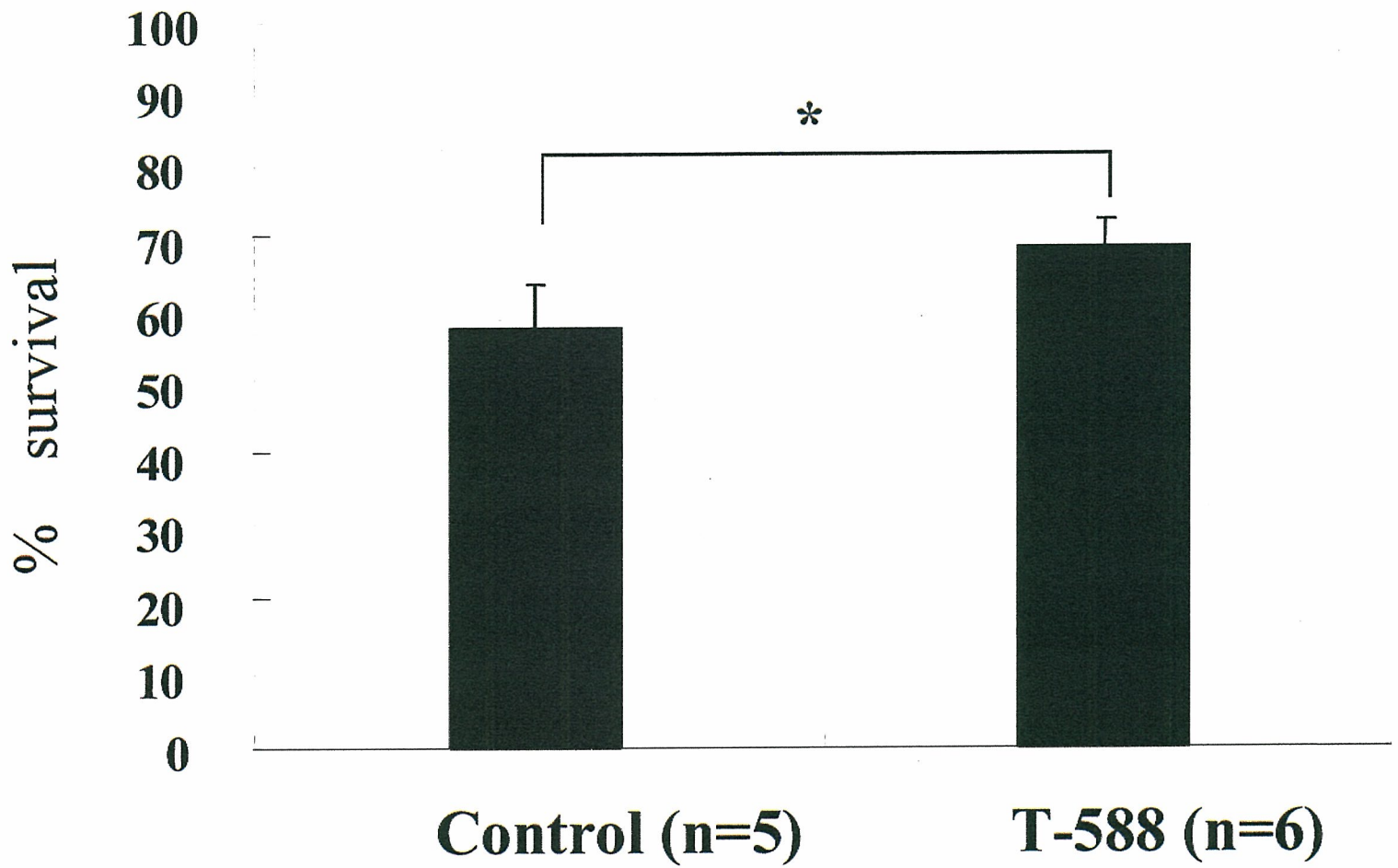
Recovery of vocal fold movement at 4 weeks after nerve crush injury. Statistically higher percentage of functional recovery was observed in the T-588-treated group, suggesting the strong functional preservation effects of T-588. Statistical comparison was performed by χ^2-test for independence (*$p<0.05$).

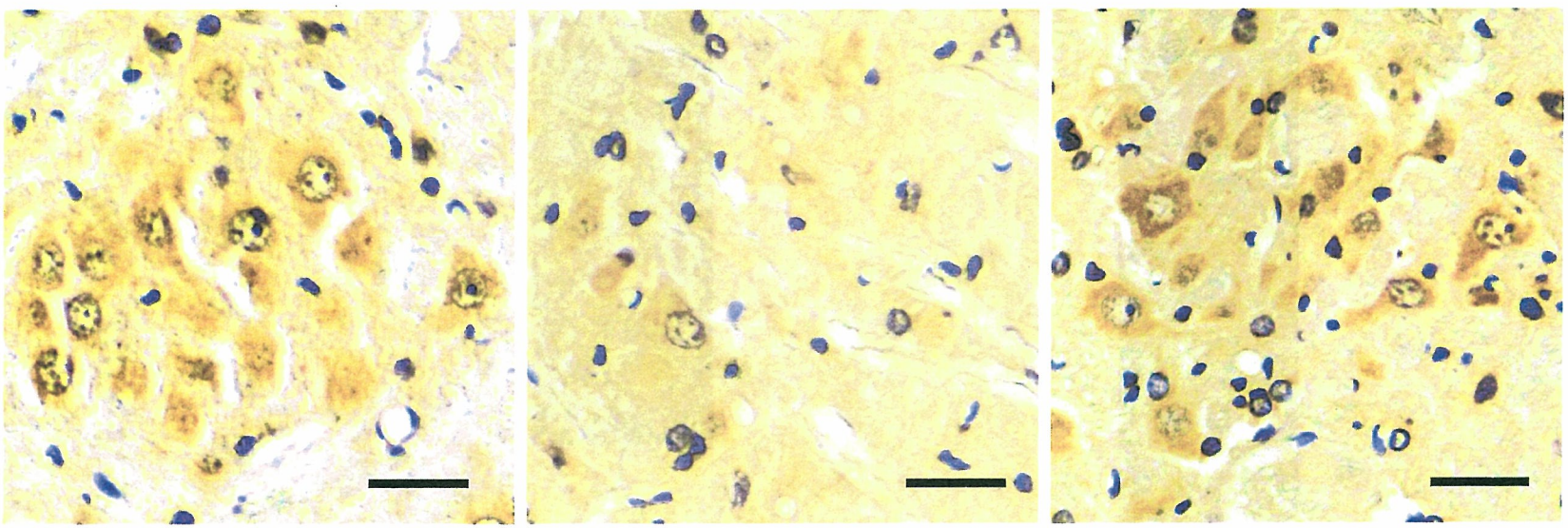
Figure 1



(a) (b) (c)

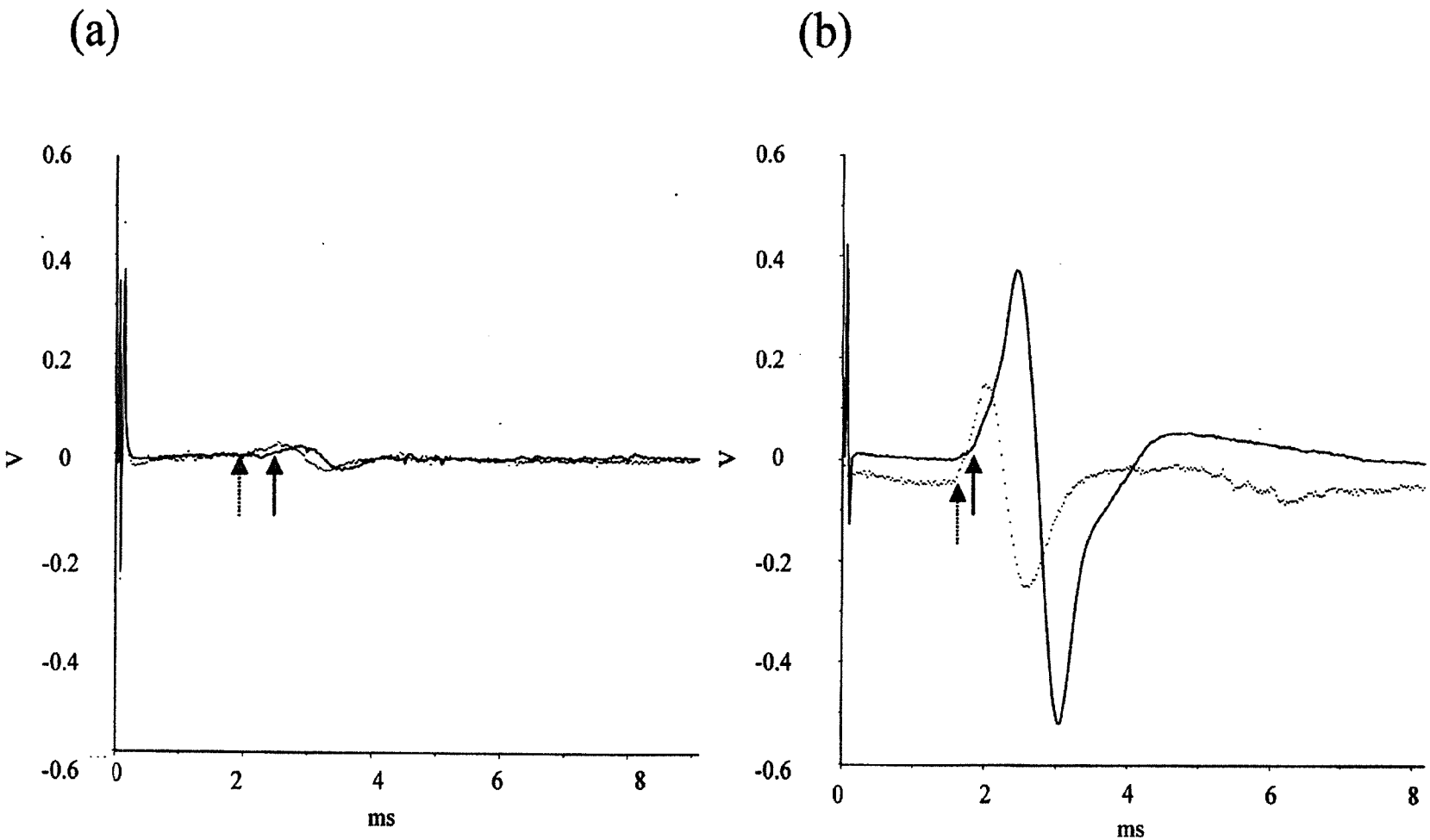
Figure 2





(a) (b) (c)

Figure 4



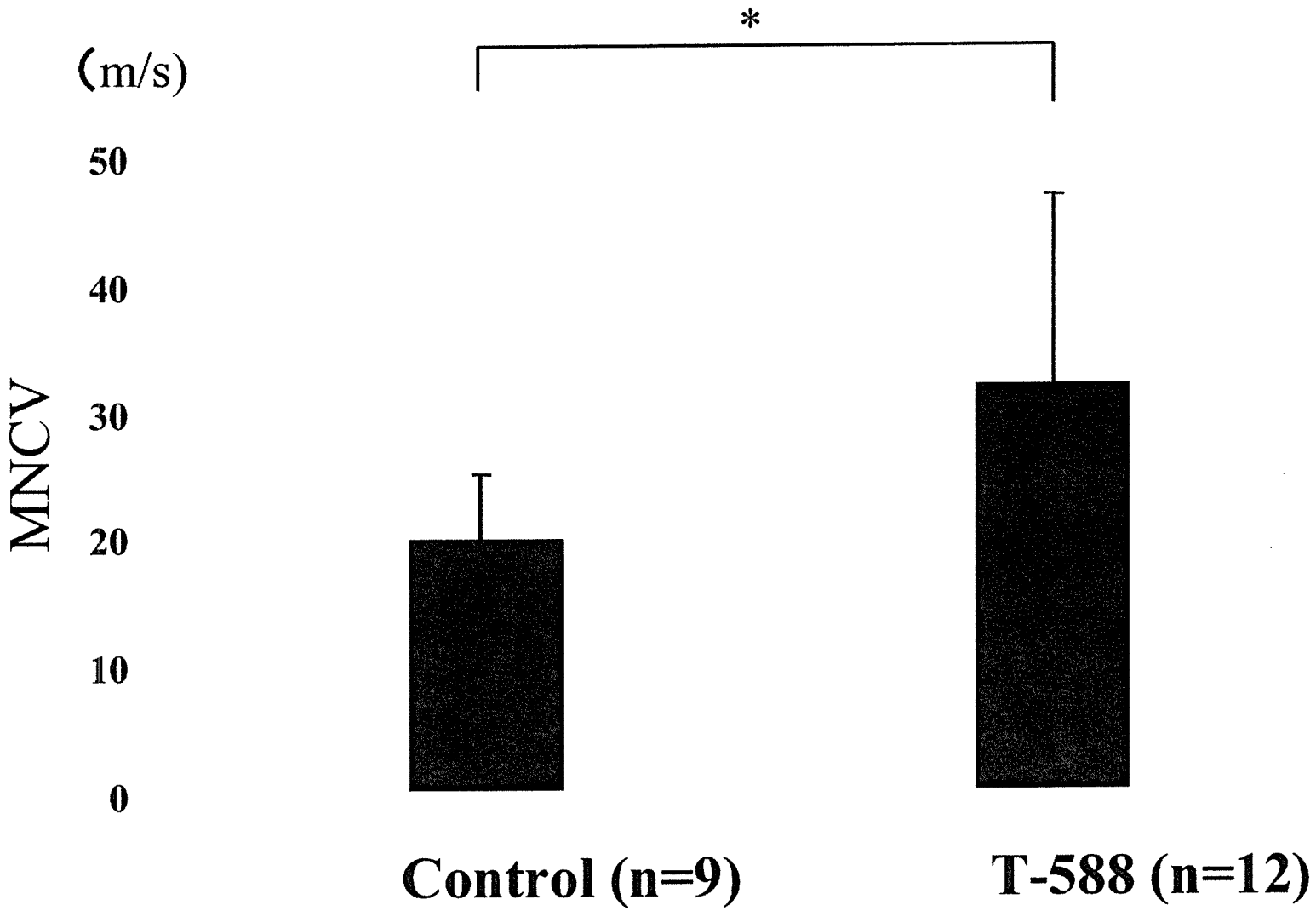

*
(m/s)
50
40
30
20
10
0
MNCV
Control (n=9)
T-588 (n=12)

Table 1

	Control (n=9)	T-588 (n=12)
recovered	2/9	9/12 *
not recovered	7/9	3/12

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