NEURAL REGENERATION RESEARCH
Volume 7, Issue 17, June 2012

www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(17):1293-1298.

Perlecan and synaptophysin changes in denervated skeletal muscle☆

Kai Ma[1, 2], Zhifeng Huang[3], Jianfeng Ma[2], Longquan Shao[4], Huiming Wang[1], Yanliang Wang[2]

1*Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Medical College of Zhejiang University, Hangzhou 310006, Zhejiang Province, China*
2*Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China*
3*School of Pharmacy, Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China*
4*Department of Stomatology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China*

Abstract

The present study observed sciatic nerve and gastrocnemius muscle changes in denervated rats using morphology methods, and assessed expression of perlecan, an extracellular matrix component, which is located at the skeletal muscle cell surface as acetylcholine esterase, as well as synaptophysin, a synaptic marker. Results showed degeneration and inflammation following transection of the sciatic nerve. In addition, the sciatic nerve-dominated skeletal muscle degenerated with mild inflammation, indicating that skeletal muscle atrophy primarily contributed to denervation-induced nutritional disturbances. With prolonged injury time (1–4 weeks post-injury), perlecan expression gradually decreased and reached the lowest level at 4 weeks, but synaptophysin expression remained unchanged after denervation. Results suggested that perlecan expression was more sensitive to denervation and reflected regional extracellular matrix changes following denervation.

Key Words

perlecan; synaptophysin; extracellular matrix; acetylcholine esterase; neuromuscular junction; denervated; skeletal muscle; sciatic nerve; neural regeneration

Abbreviations

NMJ, neuromuscular junction; AChE, acetylcholinesterase; SYN, synaptophysin

INTRODUCTION

Long-term denervation-induced skeletal muscle atrophy greatly complicates motor nerve injury[1]. Recovery of muscle atrophy and motor end plate degeneration requires sufficient time. Unfortunately, the function, quantity, and distribution of reconstructed motor end plates remains inferior to the original plate[2]. Therefore, neurological functional recovery is not favorable. Neural anastomosis or bridging does not provide acute position signals at postsynaptic membranes to sufficiently induce motor end plate reconstruction, leading to unsatisfactory results[3]. It is important to develop a method to promote ingrowth of regenerated motor nerve endings to skeletal muscle and to improve distribution of newly generated motor end plates, which could significantly provide beneficial effects of neural anastomosis and bridging. Therefore, the correlation between neuromuscular junction, which is closely related to motor nerve ending function, and motor end-plate distribution of regenerated motor nerve ending is significant for studying motor nerve regeneration distribution and functional recovery.

Kai Ma☆, M.D., Attending physician, Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Medical College of Zhejiang University, Hangzhou 310006, Zhejiang Province, China; Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China

Corresponding author: Huiming Wang, Professor, Doctoral supervisor, Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Medical College of Zhejiang University, Hangzhou 310006, Zhejiang Province, China; Yanliang Wang, Associate chief physician, Master's supervisor, Department of Oral and Maxillofacial Surgery, Affiliated Hospital of Stomatology, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China
wangmysm@yahoo.com.cn
wangyanliang@hotmail.com

Received: 2012-01-19
Accepted: 2012-04-23
(N20120112001/WLM)

Ma K, Huang ZF, Ma JF, Shao LQ, Wang HM, Wang YL. Perlecan and synaptophysin changes in denervated skeletal muscle. Neural Regen Res. 2012;7(17):1293-1298.

www.crter.cn
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.17.002

Perlecan is the major proteoglycan of the extracellular matrix. It is located in the muscle basilar membrane and aggregates in the neuromuscular junction (NMJ)[4-5]. It is also expressed at the surface of skeletal muscle cells and can bind acetylcholinesterase (AChE). The variety of perlecan sources results in an extensive distribution throughout the basilar membrane and connective tissue[6]. Different locations result in various glycosylation patterns[7]. Perlecan participates in epithelial cell growth and attachment, cartilage and bone formation and development, nerve and glial cell growth, and differentiation[8].

Perlecan can bind the extracelluar matrix, cell surface receptors, and growth factors, functioning as an adhesive to stabilize matrix structure and interactions between cells and matrix. It also mediates effects of growth factors on cell proliferation and differentiation[9-13].

A previous study reported that perlecan is involved in skeletal muscle cell regeneration following skeletal muscle injury and that it plays an important role in injury-induced muscle satellite cell activation and proliferation, as well as in the maturation and differentiation of regenerated skeletal muscle cells[14]. Perlecan-knockout mice exhibit normal muscle development, differentiation, and nerve ending formation, as well as AChE synthesis[15]. However, perlecan is barely detectable in AChE of the NMJ, indicating an important role for perlecan in AChE location within the NMJ on the surface of skeletal muscles.

Following skeletal muscle denervation, muscle cells shrink and NMJ degeneration or disappearance is observed. To further understand the role of perlecan in the NMJ under physiological and pathological conditions, as well as to investigate NMJ changes, the present study observed perlecan expression in skeletal muscle denervated rats. In addition, synaptophysin (SYN) expression was detected, which is a glycoprotein in neurons and neuroendocrine cells and an essential component of synaptic vesicles. In addition, a synaptic marker was analyzed[15-18] to compare sensitivity and specificity of perlecan expression changes.

RESULTS

Quantitative analysis of experimental animals

A total of 30 Sprague-Dawley rats were subjected to left sciatic nerve resection to establish a model of sciatic nerve injury. Samples were harvested at 1, 2, and 4 weeks after model establishment. The sciatic nerve and gastrocnemius muscle from the right side were used as controls. A total of 30 rats were included in the final analysis.

Gross observation results of sciatic nerve transection

During surgery, the gastrocnemius muscle frequently twitched when isolating the sciatic nerve, and the rats displayed restlessness. Following sciatic nerve transection, stimulation of the sciatic nerve no longer induced gastrocnemius muscle twitching. The affected limbs were unable to normally walk and limp upon awakening. After 2 weeks, the limbs at the injured side were thinner than the normal side, indicating muscle atrophy.

Pathological changes after sciatic nerve transection

Hematoxylin-eosin staining revealed edema in the nerve stem, inflammatory cell infiltration surrounding the injury site, swollen neurites, and broken and degenerated neuritis at 1 week post-injury. After 2 weeks, myelin sheath degeneration was worse at the injury site, retraction balls formed proximal to the heart at the injury site to prevent cytoplasm loss, and the distal axons decreased or even disappeared. In addition, a large number of Schwann cells were observed, accompanied by a Bungner band and lymphocyte infiltration (Figure 1). After 4 weeks, inflammation around the injury site was significantly attenuated, the nerves remained swollen, and neurite flexion was obvious.

Among the animals, there was no difference in behavior between the males and females post-injury. Sciatic nerve-dominant muscle exhibited progressive atrophy in both male and female rats. At 1 week post-injury, light microscopy revealed skeletal muscle cell thinning and flexion, cross-section area reduction, increased intercellular space, and irregularly shaped cells. Several inflammatory cells aggregated, but inflammatory vessel reaction was not obvious. By 4 weeks, skeletal muscle fiber was greatly deformed, cytoplasm was reduced, muscle fibers space increased, and vacuolus changes were observed in cells (Figure 2, supplementary Figure 1 online).

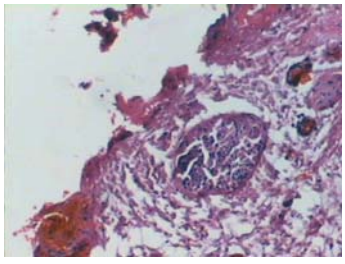

Figure 1 Histological changes in the sciatic nerve proximal to the heart at 1 week post-injury (hematoxylin-eosin staining, light microscope, × 40).

Retraction balls and lymphocyte infiltration are observed.

Perlecan and SYN expression in gastrocnemius muscle following sciatic nerve transection

In normal rats, skeletal muscle fibers were orderly

arranged, with even space among fibers, and the nuclei were distributed near the cell membrane. Immunohistochemical staining showed evenly distributed perlecan in a band-shape along the skeletal muscle cell membrane of normal skeletal muscle fibers. However, at 1 week post-injury, muscle cells shrank, and perlecan was released out of the cells and was unevenly distributed. At 2 weeks, perlecan expression significantly decreased, but was occasionally detected in the extracellular matrix surrounding skeletal muscle fibers. By 4 weeks, perlecan was not expressed, muscle cells further shrank, and skeletal muscle fibers space increased (Figure 3).

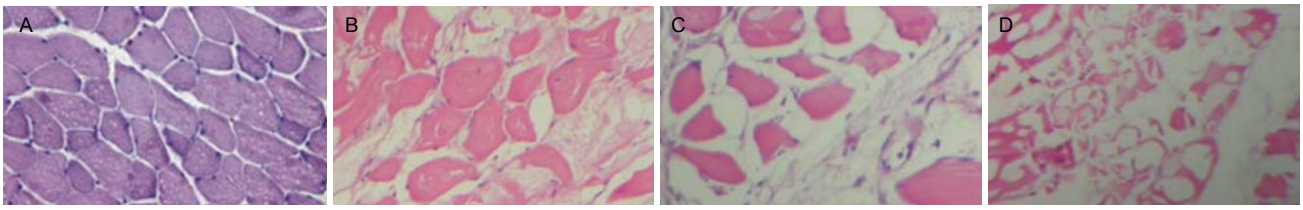

Figure 2 Pathological changes of gastrocnemius muscle following sciatic nerve injury (hematoxylin-eosin staining, light microscope, × 40).

(A) Normal gastrocnemius muscle.

(B–D) Gastrocnemius muscle at 1, 2, and 4 weeks post-injury. With prolonged injury time, skeletal muscle fiber becomes deformed, and vacuolus changes are observed in cells.

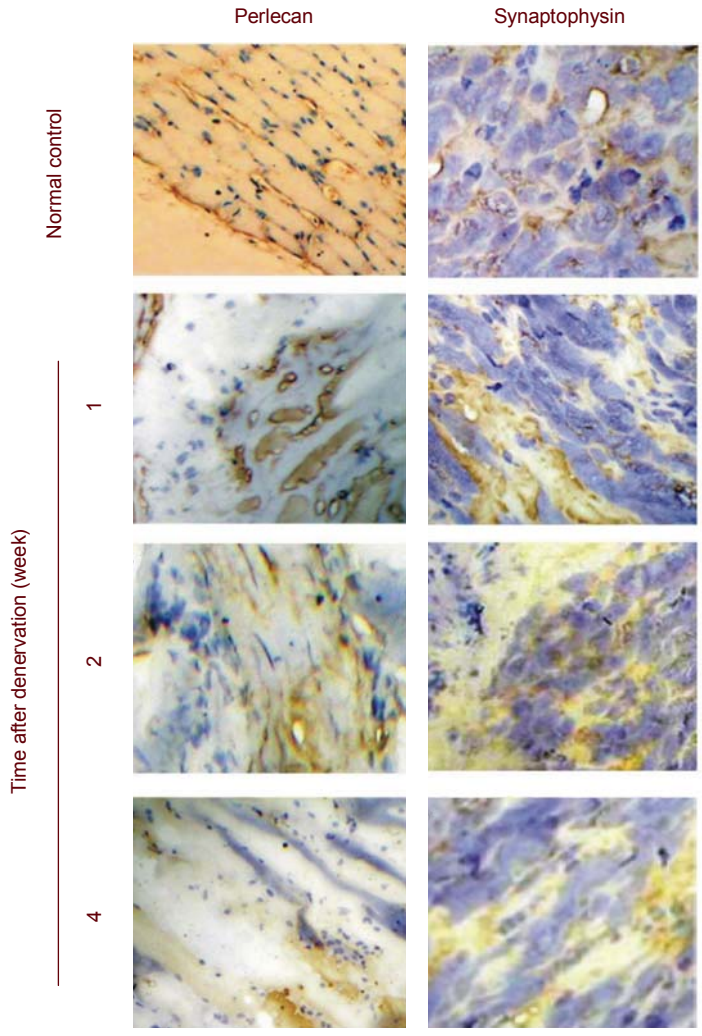

Figure 3 Perlecan and synaptophysin expression in gastrocnemius muscle at different time points following denervation (immunohistochemical staining, light microscope, × 40).

Positive products are stained brown yellow. With prolonged injury time, perlecan positive expression gradually decreases, but synaptophysin expression remains unchanged.

Image analysis showed that perlecan staining intensity gradually decreased following sciatic nerve transection, with significant differences in skeletal muscle perlecan expression at 2 and 4 weeks after denervation compared with the normal control group (gray scale value: F = 23.592, P < 0.01; Table 1).

SYN was scattered in skeletal muscle of the normal control group, but distribution density was high. Following denervation, SYN expression remained unchanged (Figure 3). Although SYN positive distribution density slightly decreased compared with the normal control, there were no significant differences at 2 and 4 weeks post-injury compared with the normal control group (gray scale value: F = 0.877, P > 0.05; Table 1).

Table 1 Perlecan and synaptophysin expression (gray scale value) in gastrocnemius muscle following denervation

Group	Perlecan	Synaptophysin
Normal control	161.6±23.8	164.3±18.3
Time after denervation		
1 week	162.9±14.4	164.1±16.8
2 weeks	178.5±13.9[a]	168.2±20.7
4 weeks	180.6±10.0[a]	166.8±21.3

Results are expressed as mean ± SD of 10 rats in each group at each time point. [a]P < 0.01, *vs.* normal control group (one-way analysis of variance).

DISCUSSION

The present study established a rat model of skeletal muscle denervation by resecting the sciatic nerve. The model was considered successful when the rats exhibited obvious regional dysfunction, combined with gross observation. In addition, the rats did not lick the incision, which is conducive to wound healing.

In the present study, perlecan and SYN expression decreased following denervation. In particular, perlecan expression rapidly and significantly decreased. Differences were induced by changes in the NMJ presynaptic and postsynaptic membranes following denervation. In the NMJ, SYN was primarily distributed in the presynaptic membrane[19], but perlecan was expressed in the postsynaptic membrane[20]. SYN release in the presynaptic vesicle was immediately terminated following nerve resection and remained until presynaptic membrane structure was broken[21]. In the postsynaptic membrane, perlecan is generated from muscle cells. Following denervation, the skeletal muscle loses a variety of neurotrophic factors and nerve impulse stimulations, resulting in nutritional disturbances and muscle atrophy. This results in the termination of perlecan synthesis and significantly decreased expression[22]. With prolonged time, molecules, including perlecan, important to AChE localization in the postsynaptic membrane significantly decrease, and perlecan expression reduction is faster than the degeneration of the regional NMJ structure. Results from the present study showed that perlecan expression was more sensitive to denervation than SYN. These results suggested that after long periods of denervation, nerves restored the dominance and related signaling molecules in the postsynaptic membrane decreased to low levels. This, in turn, likely influenced NMJ formation in the regenerated nerve, as well as localization of the motor end plate, resulting in muscular dysfunction.

In conclusion, the present study assessed perlecan and SYN expression in denervated skeletal muscle using immunohistochemistry. Results showed differences in perlecan and SYN expression. Extracellular perlecan rapidly decreased with prolonged injury, which was consistent with changes to the regional extracellular matrix following skeletal muscle denervation. However, further studies are needed to verify NMJ physiology and to determine whether the changes are consistent with decreased skeletal muscle functions. Future studies of motor nerve regeneration could utilize characteristics of perlecan to develop methods for promoting NMJ formation and motor end plate localization during nerve regeneration.

MATERIALS AND METHODS

Design

A self-controlled animal experiment.

Time and setting

The experiment was performed at Pilot Base of School of Pharmacy, Wenzhou Medical College, China in June 2011.

Materials

A total of 30 healthy, adult, Sprague-Dawley rats, with equal number of males and females and weighing 300–400 g, were provided by the Laboratory Animal Center, Wenzhou Medical College (license No. SYXK (Zhe) 2010-0150). Animal experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[23].

Methods

Establishment of denervation model, sampling and pathological observation

Animals were intraperitoneally anesthetized with 1% pentobarbital sodium (30 mg/kg). The left sciatic nerve

was isolated and transected at the inferior margin of the piriform fossa. The right side served as the control and did not undergo any treatment. Bilateral sciatic nerves and dominant gastrocnemius muscle were harvested at 1, 2, and 4 weeks after injury, fixed in 4% paraformaldehyde for 24 hours, dehydrated, cleared, paraffin-embedded, and cut into 4-μm thick sections. Pathology of sciatic nerves and dominant gastrocnemius was observed by hematoxylin-eosin staining.

Immunohistochemistry for perlecan and SYN expression in the gastrocnemius muscle

The paraffin sections were de-waxed and hydrated in gradient alcohol, incubated in freshly prepared 3% H_2O_2 at room temperature for 10 minutes to deactivate endogenous enzyme, and washed three times with distilled water for 2 minutes each. For heat-induced antigen retrieval, the sections were immersed in 0.01 M PBS (pH 6.0) and were heated in a microwave oven, followed by boiling-cooling-boiling- cooling, for two cycles in total. The sections were washed three times in distilled water for 2 minutes each, followed by incubation in primary mouse anti-perlecan (1:100) and mouse anti-SYN (1:100) monoclonal antibodies (Chemicon, Santa Cruz, CA, USA) overnight at 4°C, washed three times in 0.01 M PBS for 5 minutes each, followed by secondary goat anti-mouse IgG (1:100; SABC kit, Boster Biotechnology, Wuhan, China) at room temperature for 3 hours. The sections were then washed three times in 0.01 M PBS for 5 minutes each, colorized with diaminobenzidine, and counterstained with hematoxylin. Perlecan and SYN positive distribution was observed in denervated skeletal muscles at different time points using an Olympus light microscope (Tokyo, Japan). A HPIAS-1000 color image analysis system (Tongji University, China) was used. Prior to each measurement, gray scale was corrected using a blank area of one section, and the light source was regulated to ensure identical measurement methods for different samples. The mean brightness value of the blank area was set to 255. Four regions from one section of gastrocnemius muscle were selected to minimize personal errors. The mean gray scale value of each positive unit was obtained by automatic measurements.

Statistical analysis

Data were analyzed using SPSS version 13.0 (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Differences were compared utilizing one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank Li Zhu, School of Stomatology, Wenzhou Medical College, and Guang Liang, School of Pharmacy, Wenzhou Medical College, for technical support.

Funding: This study was supported by the National Natural Science Foundation of China, No. 30900300/C1002.

Author contributions: Kai Ma and Zhifeng Huang designed the study, integrated experimental data and wrote the manuscript. Huiming Wang and Yanliang Wang provided financial support. Longquan Shao and Jianfeng Ma provided technical support.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Wenzhou Medical College, China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

REFERENCES

[1] Stilwill EW, Sahgal V. Histochemical and morphologic changes in skeletal muscle following cervical cord injury: a study of upper and lower motor neuron lesions. Arch Phys Med Rehabil. 1977;58(5):201-206.

[2] Sobotka S, Mu L. Force characteristics of the rat sternomastoid muscle reinnervated with end-to-end nerve repair. J Biomed Biotechnol. 2011;2011:173471.

[3] Sinis N, Horn F, Genchev B, et al. Electrical stimulation of paralyzed vibrissal muscles reduces endplate reinnervation and does not promote motor recovery after facial nerve repair in rats. Ann Anat. 2009;191(4):356-370.

[4] Sanes JR, Schachner M, Covault J. Expression of several adhesive macromolecules (N-CAM, L1, J1, NILE, uvomorulin, laminin, fibronectin, and a heparan sulfate proteoglycan) in embryonic, adult, and denervated adult skeletal muscle. J Cell Biol. 1986;102(2):420-431.

[5] Bayne EK, Anderson MJ, Fambrough DM. Extracellular matrix organization in developing muscle: Correlation with acetylcholine receptor aggregates. J Cell Biol. 1984; 99(4 Pt 1):1486-1501.

[6] Saini MG, Pinteaux E, Lee B, et al. Oxygen-glucose deprivation and interleukin-1α trigger the release of perlecan LG3 by cells of neurovascular unit. J Neurochem. 2011;119(4):760-771

[7] Ha TS, Song CJ, Lee JH. Effects of advanced glycosylation endproducts on perlecan core protein of glomerular epithelium. Pediatr Nephrol. 2004;19(11): 1219-1224.

[8] Han R, Rader EP, Levy JR, et al. Dystrophin deficiency exacerbates skeletal muscle pathology in dysferlin-null mice. Skelet Muscle. 2011;1(1):35.

[9] Mashayekhi F, Sadeghi M, Rajaei F. Induction of perlecan expression and neural cell proliferation by fgf-2 in the developing cerebral cortex: an in vivo study. J Mol Neurosci. 2011;45(2):87-93.

[10] Goldberg S, Harvey SJ, Cunningham J, et al. Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane. Nephrol Dial Transplant. 2009;24(7): 2044-2051.

[11] Ellis AL, Pan W, Yang G, et al. Similarity of recombinant human perlecan domain 1 by alternative expression systems bioactive heterogenous recombinant human perlecan D1. BMC Biotechnol. 2010;10:66.
[12] Arikawa-Hirasawa E, Rossi SG, Rotundo RL, et al. Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. Nat Neurosci. 2002;5(2): 119-123.
[13] Rehm H, Wiedenmann B, Betz H. Molecular characterization of synaptophysin, a major calcium-binding protein of the synaptic vesicle membrane. EMBO J. 1986;5(3):535-541.
[14] Gould VE, Wiedenmann B, Lee I, Schwechheimer K,Synaptophysin expression in neuroendocrine neoplasms as determined by immunocytochemistry. Am J Pathol. 1987;126(2):243-257.
[15] Colasante C, Brouard MO, Pecot-Dechavassine M. Synaptophysin (p38) immunolabelling at the mouse neuromuscular junction. Neuromuscul Disord. 1993;3(5-6): 395-400.
[16] Noonan DM, Fulle A, Valente P, et al. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. J Biol Chem. 1991;266(34): 22939-22947.
[17] Joseph SJ, Ford MD, Barth C, et al. A proteoglycan that activates fibroblast growth factors during early neuronal development is a perlecan variant. Development. 1996; 122(11):3443-3452.
[18] Casar JC, Cabello-Verrugio C, Olguin H, et al. Heparan sulfate proteoglycans are increased during skeletal muscle regeneration: requirement of syndecan-3 for successful fiber formation. J Cell Sci. 2004;117(Pt 1): 73-84.
[19] Gordon SL, Leube RE, Cousin MA. Synaptophysin is required for synaptobrevin retrieval during synaptic vesicle endocytosis. J Neurosci. 2011;31(39):14032-14036.
[20] Singhal N, Martin PT. Role of extracellular matrix proteins and their receptors in the development of the vertebrate neuromuscular junction. Dev Neurobiol. 2011;71(11): 982-1005.
[21] Gordon SL, Leube RE, Cousin MA. Synaptophysin is required for synaptobrevin retrieval during synaptic vesicle endocytosis. J Neurosci. 2011;31(39):14032-14036.
[22] Sigoillot SM, Bourgeois F, Lambergeon M, et al. ColQ controls postsynaptic differentiation at the neuromuscular junction. J Neurosci. 2010;30(1):13-23.
[23] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

(Edited by Gao HW, Sun H/Su LL/Song LP)