Effect of FTY720 on the Tissue Microenvironments of Acute Spinal Cord Injury

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Abstract: Objectives: To observe the effect of FTY720 on the changes of tissue microenvironment after acute spinal cord injury (ASCI) in rats. Methods: A total of 168 female SD rats were randomly divided into A, B and C groups, with 56 rats in each group. In group A (Sham-operation group), only T9 laminectomy was performed without spinal cord injury, and 0.3 ml normal saline was given by gavage immediately after suture. Group B (control group) was given 0.3 ml normal saline by gavage, group C (treatment group) was given 0.3 ml FTY720 diluted in 3mg/kg normal saline by gavage. The rats were sacrificed at 6h, 12h, 24h, 72h, 7d and 21d after operation. The injured spinal cord (the corresponding part of group A) was taken for ultrathin section, and HE staining was used to observe the necrosis of the spinal cord, inflammatory cell infiltration, glial scar formation, and the size of the syringomyelia in each group. The ratio of syringomyelia area to spinal cord area was calculated 21 days after injury. SPSS 13.0 software was used for statistical analysis. Results: HE staining showed that the morphology of the spinal cord in group A was normal at each time point: At 12h to 48h after operation, progressive edema of the spinal cord and liquefaction necrosis of the injured central area were observed, accompanied by inflammatory cell infiltration, mainly neutrophils, lymphocytes and monocytes. At 12h and 72h after operation, the degree of inflammatory cell infiltration in group B was significantly higher than that in group C ($P<0.05$). The degree of lymphocyte infiltration in group C was significantly lower than that in group B 12 hours after operation ($P< 0.05$). At 72 hours after operation, the central area of the injury had formed an unorganized structure cavity, and a large number of inflammatory cells infiltrated around the cavity, mainly microglia/monocytes. The number of glial scar cells in group B was significantly higher than that in group C ($P < 0.05$). The syringomyelia formed 21 days after operation. The syringomyelia ratio in group B was significantly higher than that in group C ($P<0.05$). Conclusions: FTY720 can significantly improve neurological function in rats after ASCI possibly by inhibiting the inflammatory response after spinal cord injury, thereby reducing the secondary injury of the spinal cord.

Keywords: FTY720; Neural Apoptosis; Acute Spinal Cord Injury

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1. Introduction

Acute spinal cord injury (ASCI)[1] is extremely serious, with an annual incidence of about 20-40 /million people in the world[2]. The disability rate remains high[3]. Acute spinal cord injury can be divided into primary injury and secondary injury. A large number of studies have shown that the outcome of ASCI depends not only on the degree of primary injury, but also on the development of secondary injury. Therefore, the treatment of spinal cord injury is mainly aimed at secondary injury. In recent years, with the deepening of the research on a series of biochemical reactions triggered by secondary injury[4-6], the impact of autoimmune factors on spinal cord injury has received increasing attention. Improving the microenvironment of spinal cord injury and promoting functional recovery through immunosuppressive therapy has become a new approach for the treatment of spinal cord injury.[7]

After methylprednisolone[8, 9], a few immunosuppressive drugs have been found to be effective in the treatment of acute spinal cord injury[10, 11], but the systemic side effects of immunosuppressive drugs have seriously affected their clinical application.FTY720[12, 13], chemically named 2-amino-2·[2(4-octylphenyl)ethyl]-1 ， 3-propanediol hydrochloride, is synthesized by chemical modification of ISP-1 from the culture medium of Cordyceps sinensis. It is a new immunosuppressive drug different from other immunosuppressive drugs. FTY720 has little toxic and side effects on human body, and there is no significant decrease in peripheral blood neutrophils. It does not cause obvious damage to liver and kidney function, and can effectively play the role of immune regulation while maintaining the immune function of patients[14]. Recent studies have shown that FTY720 has a certain therapeutic effect on neuronal injury in brain tissue[15, 16], but the specific mechanism is still unclear. Whether FTY720 has a protective effect on neurons in acute spinal cord injury is still unknown. In this study, based on the establishment of an experimental acute spinal cord injury animal model, FTY720 was used to treat the injured animals, and the protective effect of a new immunosuppressant on spinal cord injury was explored from the perspective of neuronal microenvironment and cell apoptosis.[17-19]

2. Materials and methods

2.1 Materials

2.1.l Experimental animals

84 female Sprague-Dawley rats, weighing 180g--220g, were provided by the Animal Experimental Center of Dalian Medical University (SCXK(Liao) 2008.0002).The treatment of animals in the process of animal ethics experiments should refer to the Guiding Opinions on the Good Treatment of Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China in 2006.

2.1.2 Reagents and instruments

FTY720: purchased from Wuhan Yuancheng Technology Development Co., LTD.

Caspase-3 antibody: Caspase3(CPP32)Ab-4 was produced by NeoMarkers For Lab Vision Corporation (USA).

Tunel kit: Produced by Roche Diagnostics GmbH Mannheim, Germany.

SP kit (ready-to-use type) : contains H_20_2, 5% sheep serum, sheep anti-rabbit polyclonal antibody, peroxidase.Purchased from Beijing Zhongshan Jinqiao Biotechnology Co., LTD.

Allen's Animal Spinal Cord Crusher.

2.2 Method

2.2.1 Experimental grouping and animal model preparation

A total of 84 female SD rats were randomly divided into 3 groups: Sham-operation group (group A), model group (group B) and FTY720 treatment group (group C), including 14 rats in group A, 35 rats in group B and 35 rats in group C. The rats in the model group and the FTY720 treatment group were anesthetized intraperitoneally with 2% chloral hydrate (60mg/kg) and placed in the prone position. The skin was prepared with T9 as the center, and the skin was routinely sterilized. The spinous processes of T9 and T10 and the corresponding lamina were exposed, and the back and sides of the spinal cord were fully exposed by biting the T9 and T10 spinous processes and the corresponding lamina with a rouge. Under aseptic condition, the spinal cord of rats was hit by modified A11en's method. If the rats showed tail wagging reflex and severe contraction of lower limbs after impact, the model was successfully established. After the impact, the wound was sutured aseptically. In the Sham-operation group, only T9 and T10 pushing plate resection was performed without hitting the spinal cord, and the rest were the same as the model group. After modeling, the FTY720 treatment group was diluted with 3mg/kg normal saline and 0.3 ml was given by gavage. The Sham-operation group and the model group were given the same amount of normal saline by gavage. After operation, the rats were fed in cages with light and natural ventilation. After operation, urination was performed 2-3 times a day by manual pressing. The food intake, wound healing, and adverse reactions were observed.

2.2.2 Sampling and section preparation

The rats in each group were sacrificed at 6h, 12h, 24h, 48h, 72h, 1w, and 3w after operation, and 5 rats in each group were sacrificed at each time point. The rats were anesthetized intraperitoneally with 2% chloral hydrate (60mg/kg), then the hearts were perfused with 50ml of 4% paraformaldehyde 0.1M PH7.4 PBS solution. The injured spinal cord was about lcm long and cut into two segments from the center of the injured spinal cord. After 24 hours, the injured spinal cord was embedded in paraffin and serially sectioned with a thickness of 4μm. One section was taken every 10 minutes for HE staining. Ten sections from each specimen were taken for HE staining to observe the destruction of spinal cord tissue, inflammatory cell infiltration, the size of the syringomalacia, glial scar formation and repair of the spinal cord.

2.2.3 Detection indicators

2.2.3.1 Pathological observation of spinal cord microenvironment

The tissue sections were taken for HE staining at 6h, 12h, 24h, 72h, 1w and 3w after operation. The HE stained sections were taken by a digital camera and the images were input into a computer. The high power field images of four non-repeated spinal cord injury areas at l2h, 72h and 1w after injury were selected to calculate the cell density as M, M= the sum of the area of inflammatory nuclei in each specimen/the sum of the area of each specimen observed area ×1 00%. Inflammatory cell infiltration and glial scar formation were observed. The lymphocyte ratio at PIH 12 was calculated and recorded as L (L= lymphocyte count/total cell count ×100%).At 3w after injury, the ratio of syrinx area to total spinal cord area was calculated from the section at 20 × low power and recorded as S, S= syrinx area/spinal cord area ×100 %.

Procedure of HE staining

①Deparaffinized to water:

②Staining with hematoxylin for 2min.

③Wash back blue for 10min.

④Dye with eosin for 1.2min.

⑤Rinse with running water for 2min.

⑥Dehydrate, clear and seal

2.2.4 Image processing methods

The data were analyzed and processed by Image· pro plus 6.0 medical image analysis system.

2.2.5 Statistical methods

SPSSl3.0 software was used for statistical processing, and the data were expressed as mean 4-standard deviation. One-way analysis of variance was used for comparison among multiple groups.t test was used for comparison between the two groups, and $P<0.05$ was considered the difference was statistically significant.

3. Results

3.1 Histomorphological observation of spinal cord injury tissues by HE staining

3.1.1 Morphological changes in Sham-operation group (group A), model group (group B) and FTY720 treatment group (group C) at 6 hours are shown in Figure l Morphological changes

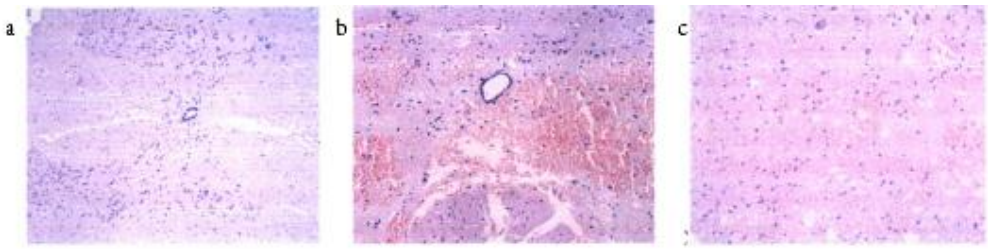

Figure 1. a: Sham-operation group (group A) b: model group (group B) c:FTY720 treatment group (group C).

In group A, the morphology of spinal cord was intact at each time point, and no destruction or liquefaction necrosis was observed. In group B and group C, obvious intraspinal hemorrhage and local hematoma were observed 6 hours after acute spinal cord injury, but the general shape of the spinal cord was still intact, the morphology of neurons was basically normal, and no obvious inflammatory cell infiltration was observed.

3.1.2 The morphological changes of the model group (group B) and FTY720 treatment group (group C) at 12 hours are shown in Figure 2

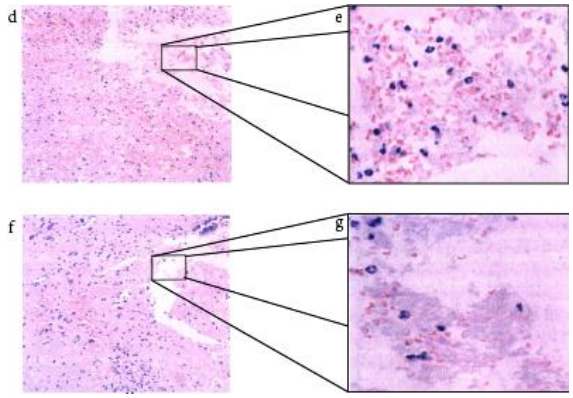

Figure 2. d, e: Model group (group B) f, g:FTY720 treatment group (group C).

At 12 hours after injury, liquefaction necrosis and inflammatory cell infiltration were observed in the spinal cord of group B and group C. Under low power field, the spinal cord tissue in group B showed obvious liquefaction necrosis, the central area of the tissue disintegrated and disappeared, and formed large cavities, and the internal hemorrhage was still obvious. The morphology of the spinal cord tissue in group C was mostly preserved, and the degree of liquefaction necrosis of the spinal cord was significantly lighter than that in group B, and small cavities were formed in some areas, and the internal hemorrhage was still obvious. In high power field, the infiltration of inflammatory cells in group B was mainly neutrophils, monocytes and lymphocytes. The infiltration of inflammatory cells in group C was mainly composed of neutrophils and monocytes, and lymphocytes were almost not seen.

3.1.3 The 24-hour morphological changes of the model group (group B) and FTY720 treatment group (group C) are shown in Figure 3

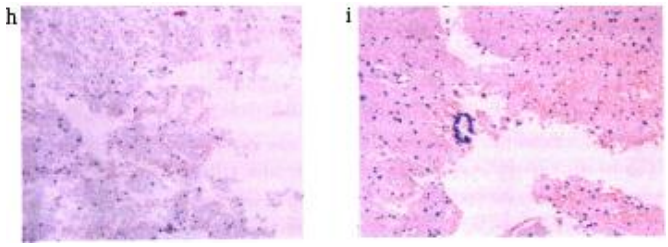

Figure 3. h: Model group (group B) i:FTY720 treatment group (group C)

At 24 hours after injury, the degree of liquefaction necrosis of spinal cord tissue in group B and group C was further aggravated, and group B was still more serious than group C. The number of neutrophils infiltration in the syringomyelia was gradually reduced, the number of microglia/monocytes around the syringomyelia was gradually increased, and the spinal cord hematoma was alleviated.

3.1.4 The morphological changes of model group (group B) and FTY720 treatment group (group C) at 72 hours are shown in FIG. 4

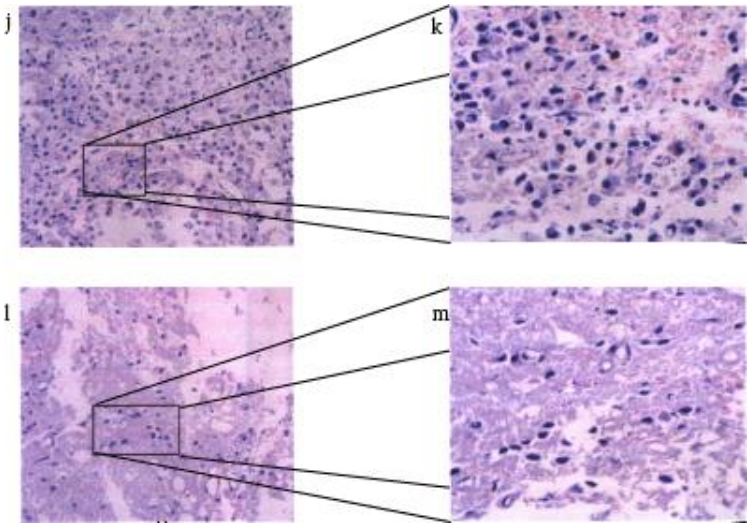

Figure 4. j, k: model group (group B) l, m:FTY720 treatment group (group C).

At 72 hours after injury, the myelomalacia area was further enlarged under low power field, and it was still more severe in group B than in group C. In group B2 and C2, the inflammatory cell infiltration was mainly microglia/monocytes, mainly located around the central area of the injury, but almost no neutrophils and lymphocytes were seen. The hemorrhage in the spinal cord still existed.

3.1.5 The morphological changes of model group (group B) and FTY720 treatment group (group C) at 1 w are shown in FIG. 5

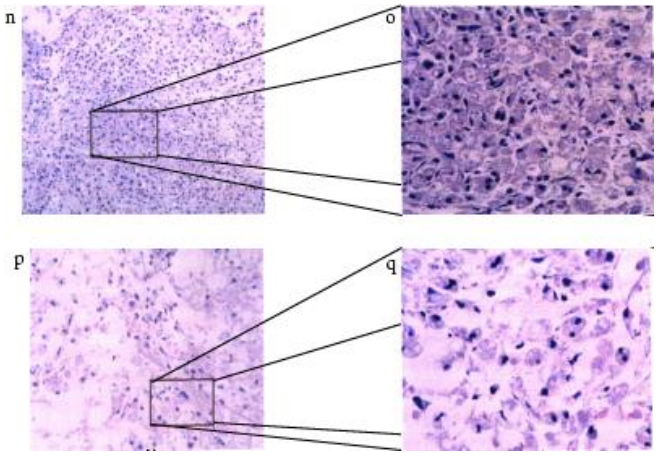

Figure 5. n, o: model group (group B) p, q:FTY720 treatment group (group C)

1 w after injury, the degree of liquefaction necrosis of the spinal cord tissue in groups B and C remained unchanged under low power field, and the hematoma in the spinal cord was almost absent. In group B, the degree of microglia/monocytes infiltration around the central area of spinal cord injury was further increased, oligodendrocytes and astrocytes proliferated, and the tissue structure was dense. In group C, the degree of microglia/monocyte infiltration around the central area of spinal cord injury was not significantly increased compared with that at 72 hours, and the proliferation of oligodendrocytes and astrocytes was not obvious.

3.1.6 The morphological changes of the model group (group B) and FTY720 treatment group (group C) at 3 weeks are shown in Figure 6

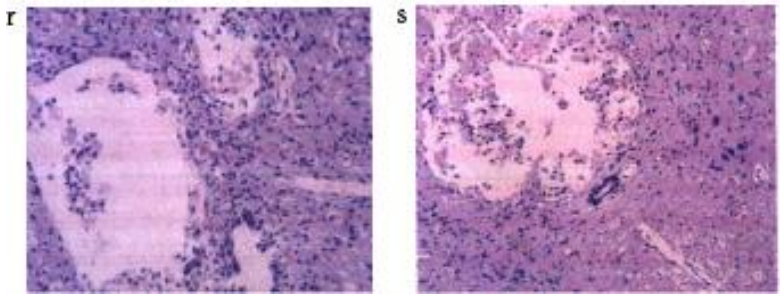

Figure 6. r: Model group (group B) s:FTY720 treatment group (group C)

At 3 weeks after injury, obvious cavities were formed in the injury center of group B and group C. In group B, there was a dense glial scar surrounding the syringomyelia and a wide transitional zone between the syringomyelia and normal spinal cord tissue. In group C, normal spinal cord tissue was observed around the syringomyelia, and there was almost no glial scar around the syringomyelia.

4. Discussion

4.1 Effect of FTY720 on tissue emblem environment in rats with acute spinal cord injury

More and more attention has been paid to the role of autoimmune response in ASCI.A large number of studies have shown that after ASCI, there will be a transient aggregation of neutrophils in the injured tissue, which means the beginning of the immune response. Antigen-specific T lymphocytes (T-LC) and antigen-nonspecific macrophage cells (MC) play a major role in the autoimmune response. The use of immunosuppressants to inhibit the local inflammatory response and improve the microenvironment of the injured neurons has become one of the new ideas for the treatment of spinal cord injury.

Current studies[20-22] have shown that FTY720 can not only promote the homing of lymphocytes to lymph nodes, but also effectively reduce the infiltration of T lymphocytes into local tissues. Therefore, FTY720 can reduce the inflammatory response by reducing the infiltration of local immune cells. In this experiment, HE staining showed that in the model group (group B), obvious blood could be seen in the spinal cord at 6 hours after acute spinal cord injury, but the morphology of the spinal cord was still roughly intact, the morphology of neuronal cells did not change significantly, and the infiltration of inflammatory cells was not obvious. However, at 12 hours after injury, liquefaction necrosis and infiltration of inflammatory cells were observed in the spinal cord tissue, mainly neutrophils, monocytes and lymphocytes. From 24 to 72 hours after injury, the liquefaction and necrosis of the spinal cord tissue were progressively aggravated. The infiltration of neutrophils and lymphocytes gradually decreased or almost disappeared, while the infiltration of microglia/monocytes gradually increased. At 1 week after injury, the infiltration of microglia/monocytes was predominant around the liquefaction necrosis area of the spinal cord, with a large number of oligodendrocytes and astrocytes. Obvious cavities could be seen in the center of the spinal cord injury area 3 weeks after injury. Although the changes of microenvironment in FTY720 treatment group (group C) were basically the same as those in model group (group B) after spinal cord injury, the degree of inflammatory

cell infiltration, especially lymphocyte infiltration, and the size of glial scar and cavity in FTY720 treatment group (group C) were significantly smaller than those in model group (group B) at all time points except 6 hours after injury. The results of quantitative analysis showed: In the model group (group B), the cell density of inflammatory cell infiltration and the cell ratio of lymphocyte infiltration at 12 hours after SCI, the density of microglia/monocytes at 72 hours, the density of glial scar cells around the first week, and the ratio of cavity area to spinal cord area at 3 weeks were significantly higher than those in the FTY720 treatment group (group C). And the difference was statistically significant ($P<0.05$).

According to the above results analysis, it can be concluded that: FTY720 alleviates secondary injury after acute spinal cord injury by reducing the infiltration of inflammatory cells and the release of related cytokines (especially T lymphocytes), reducing the area of glial scar and cavity, and retaining more normal spinal cord tissue, thus improving the microenvironment of acute spinal cord injury tissue and protecting neurons.

5. Conclusion

FTY720 can reduce the secondary injury of spinal cord by reducing the infiltration of inflammatory cells in rats with acute spinal cord injury.

References

[1] Cortez R, Levi AD. Acute spinal cord injury [J]. Critical Care Clinics, 1987, 3(2): 441-697.

[2] Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms [J]. Journal of Neurosurgery, 1991, 75(1): 15-26.

[3] Tyor WR, Avgeropoulos N, Ohlandt G, et al. Treatment of spinal cord impact injury in the rat with transforming growth factor-beta [J]. Journal of the Neurological Sciences: Official Bulletin of the World Federation of Neurology, 2002, (1/2): 200.

[4] Merola A, et al. Attenuation of CNTF in ASCI treated with intravenous methylprednisolone [J]. The Spine Journal, 2002, 2(Supplement): 109.

[5] Bracken MB, Shepard MJ, Collins W, et al. Bracken M B, Shepard M J, Collins W F et alMethylprednisolone or naloxone treatment after acute spinal cord injury: I-year follow-up data. Results of the second National Acute Spinal Cord Injury Study, 1992. J Neurosurg 76: 23-31 [J]. Journal of Neurosurgery, 1992, 76(1): 23-31.

[6] Bracken M. A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal cord injury [J]. N Engl J Med, 1990, 322.

[7] Beattie MS, Hermann GE, Rogers RC, et al. Cell death in models of spinal cord injury [J]. Progress in brain research, 2002: 137.

[8] John HR. Methylprednisolone for the Treatment of Acute Spinal Cord Injury: Point [J]. Neurosurgery, 2014, 61 Suppl 1(Suppl 1): 32.

[9] Bracken MB, Shepard MJ, Collins WF, et al. Methylprednisolone or naloxone treatment after acute spinal cord injury: I-year follow-up data [J]. Journal of Neurosurgery, 1992, 76(1): 23-31.

[10] Lambrechts D, Carmeliet P. VEGF at the neurovascular interface: therapeutic implications for motor neuron disease [J]. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2006, 1762(11–12): 1109-21.

[11] Ibarra A, Correa D, Willms K, et al. Effects of cyclosporin-A on immune response, tissue protection and motor function of rats subjected to spinal cord injury [J]. Brain Research, 2003, 979(1-2): 165-78.

[12] Fujita, Inoue, Yamamoto, et al. Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite [J]. The Journal of antibiotics, 1994.

[13] Kappos L, Antel J, Comi G, et al. Oral fingolimod (FTY720) for relapsing multiple sclerosis [J]. N Engl J Med,

2006, 355(11): 1124-40.

[14] Budde K, Schmouder R L, Brunkhorst R, et al. Budde, K. et al. First human trial of FTY720, a novel immunomodulator, in stable renal transplant patients. J. Am. Soc. Nephrol. 13, 1073-1083 [J]. Journal of the American Society of Nephrology, 2002, 13(4): 1073-83.

[15] Zhang Z, Fauser U, Schluesener HJ. Early attenuation of lesional interleukin-16 up-regulation by dexamethasone and FTY720 in experimental traumatic brain injury [J]. Neuropathology and Applied Neurobiology, 2008.

[16] Zhang, Z, et al. FTY720 attenuates lesional interleukin-17+ cell accumulation in rat experimental autoimmune neuritis [J]. Neuropathology & Applied Neurobiology, 2009.

[17] Brinkmann, V. The Immune Modulator FTY720 Targets Sphingosine 1-Phosphate Receptors [J]. Journal of Biological Chemistry, 2002, 277(24): 21453-7.

[18] Schmouder R, Serra D, Wang Y, et al. FTY720: placebo-controlled study of the effect on cardiac rate and rhythm in healthy subjects [J]. Journal of Clinical Pharmacology, 2013, 46(8).

[19] Brinkmann V, Billich A, Baumruker T, et al. Erratum: Fingolimod (FTY720): Discovery and development of an oral drug to treat multiple sclerosis (Nature Reviews Drug Discovery (2010) 9 (883-897)) [J]. 2010.

[20] Li X K, Tamura A, Fujino M, et al. Induction of lymphocyte apoptosis in rat liver allograft with ongoing rejection by FTY720 [J]. Clinical & Experimental Immunology, 2010, 123(2): 331-9.

[21] López-Vales R, García-Alías G, Forés J, et al. FK506 reduces tissue damage and prevents functional deficit after spinal cord injury in the rat [J]. Journal of Neuroscience Research, 2010, 81(6): 827-36.

[22] Nagahara Y, Ikekita M, Shinomiya T. Immunosuppressant FTY720 induces apoptosis by direct induction of permeability transition and release of cytochrome c from mitochondria [J]. Journal of Immunology, 2000, 165(6): 3250-9.